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Studies on the Nutritive Value of Milk

I. The Deficiencies of an Exclusive Milk Diet and How to Overcome Them

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STUDIES ON THE NUTRITIVE VALUE OF MILK

I. The Deficiencies of an Exclusive Milk Diet and How to Overcome Them

W. E. KRAUSS

INTRODUCTION

As one of the steps in a study of the food value of milk as affected by various rations fed to cows, rats were fed a diet consisting entirely of fresh, raw, liquid, whole milk. It was thought that a more accurate measure of the total nutritive effect, aside from reproduction, of milk from various sources could be obtained in this way, inasmuch as no foreign material whatsoever would be knowingly introduced. Furthermore, any influence that a basal ration might exert on the inherent properties of fluid milk would be eliminated.

It was not expected that data on reproduction could be obtained since investigators had shown that reproduction in rats on diets consisting entirely of milk, milk and bread, or milk and rice was very unsatisfactory, young rarely being produced beyond the second generation and such young being extremely anemic. Investigators generally have considered it essential to carry rats through four or five generations in order to measure adequately the value of a diet for reproduction. Consequently, in the beginning this study was confined to growth and physical condition. It was anticipated, owing to the small amount of iron furnished by an exclusive milk diet, that anemia would be encountered but not until sufficient data had been obtained to measure the milk's value for growth. However, it soon became apparent that the exclusive milk method of feeding could not be employed. Animals subjected to such a feeding régime soon developed severe anemia and died.

In order to carry on the study as originally planned, the factor of anemia had to be eliminated through supplementary feeding without materially affecting the original chemical or physical properties of the milk diet. This report covers the experimental work involved in the solution of this problem, together with information obtained in following the original problem through several of its many ramifications.

HISTORICAL

Role of Iron in Anemia

A study of anemia belongs primarily in the field of medicine, but discoveries made in recent years have opened the field to the student of nutrition. Of the many types of anemia known, three, hemorrhagic, pernicious, and nutritional, have received the greatest attention recently. Early studies in anemia centered around iron alone, and it is therefore necessary, in preparing a suitable background for a discussion of nutritional anemia, to trace briefly the history of iron in nutrition.[1]

Anemia and iron have been associated closely, both popularly and in most of the great number of investigations that have been concerned with the disease. This is probably due to the fact that iron comprises from 0.4 to 0.5 per cent of hemoglobin (although sulfur is present in greater proportion) and that when hemoglobin is broken up it yields a simple protein, globin, and an iron-containing radical. It is generally known that the union of oxygen and hemoglobin is a very vital body process and that iron is a good carrier of oxygen. Iron is found not only in the red corpuscles, but very generally in active cells, both animal and vegetable. It is natural, then, that much of the work dealing with blood conservation and blood regeneration should be concerned with iron.

It was once thought that iron existed in food as an oxide or phosphate and that hemoglobin was formed in the body by the combination of protein with inorganic iron. According to Sherman, "This idea seemed to be supported by the successful use of inorganic iron in the treatment of anemia. Results of work done between 1854 and 1884 threw doubt upon the utilization of inorganic iron for the production of hemoglobin. To harmonize these results with clinical experience it was suggested that the inorganic iron acted by absorbing the hydrogen sulfide found in the intestine, thus protecting the food iron from waste." About this same time, the view was also held that medicinal iron acts by stimulating the absorbing membrane, thus increasing the absorption of food iron.

Bunge was one of the doubters of the value of inorganic iron; consequently, he took up a study of the iron compounds of food materials and, in 1884, came to the conclusion that iron occurred in food solely in the form of complicated organic compounds which are

[1]For fuller discussions see Sherman, H. C., Iron in food and its function in nutrition. Bull. 185, Office of Experiment Stations, U. S. Dept. of Agriculture (1907); and Sherman, H. C., Foods and nutrition, 3d Edition, pp. 332-348 (1927).

built up by plants. According to his theory the iron was absorbed and assimilated in this form, and from these compounds hemoglobin was produced.

This stimulated further research and for the next 15 years much work along this line was done. The results showed that both inorganic and organic iron were utilized in much the same way by the body, but that iron salts were not equivalent in nutritional value in actual feeding trials to food materials naturally rich in iron.

It must be remembered that while many of these early experiments were very carefully planned and performed there was no knowledge of the vitamins or of the importance of ratios between various mineral elements.

Role of Other Substances in Anemia

Many studies have been made on the influence of various food factors, other than iron, on hemoglobin regeneration, from which it must be concluded that diet is of primary importance in the treatment of certain anemias. Very little of this work, however, has been concerned with nutritional anemia in the true sense. Anemia induced by hemorrhage, for example, is not comparable to that brought about by faulty diet. In one instance, the hemoglobin is reduced mechanically; in the other, the reduction in hemoglobin is due to the absence of a factor or factors essential for hemoglobin formation. Pernicious anemia, likewise, falls into another category. It is interesting, and perhaps of considerable significance, that some substances have been shown to be beneficial in all three types of anemia.

Nutritional Anemia

That the iron content of milk is very low was first noted by Bunge in 1874 and confirmed by him in 1889 (8). Abderhalden (1), in 1900, was the first to show that an animal fed on an exclusive milk diet will develop anemia. Contemporary interest in this type of anemia was stimulated by the work of Hart, Steenbock, Elvehjem, and Waddell (28), who found that, when rabbits were made anemic by feeding whole milk plus sodium citrate, the addition of inorganic iron as ferric oxide did not remedy the anemia, although the animals were enabled to make good growth for some time. When cabbage was added to this diet anemia was not as prevalent, and, when milk, inorganic iron, and cabbage were fed together, there was no tendency toward anemia. Furthermore, when an iron-free alcoholic extract of dried cabbage or of yellow corn meal was fed in addition to milk and iron, no anemia developed.

Contrary to the Wisconsin results, Mitchell and Schmidt (47) found iron to be effective in curing nutritional anemia in second generation, anemic rats, the degree of effectiveness depending upon the solubility, rather than the nature (organic or inorganic) of the iron. As a result of this and subsequent work (48), iron salts were classified, according to their hemoglobin regenerating potency, as follows:

Good	Ferric acetate Ferric albuminate Ferric chloride Ferric citrate
Fair	Peptonized ferric oxide Saccharated ferric oxide Saccharated ferrous carbonate Ferrous iodide
Poor	Ferric oxide Ferrous carbonate Ferric potassium tartrate Ferrous lactate Ferrum reductum Ferrous sulfate

That the deficiency leading to nutritional anemia is inorganic in nature was shown by Hart, Elvehjem, Waddell, and Herrin (27), who corrected the disease in rabbits with the ash of lettuce, cabbage, beef liver, spleen, marrow, or yellow corn. A sample of C. P. ferrous sulfate was effective, but upon further purification its power was lost. This suggested the possibility of some active substance in ordinary iron preparations and pointed to a possible fallacy in the work of Mitchell and Schmidt.

Waddell, Elvehjem, Steenbock, and Hart (59) obtained but slight beneficial effect from the administration of inorganic iron salts to anemic rats. The chloride, sulfate, acetate, citrate, and phosphate were fed so as to furnish 0.5 mg. of iron daily. However, when the ashed residues from dried beef liver, dried lettuce, and yellow corn, and acid extracts of the same, were fed so as to furnish 0.5 mg. of iron daily, the anemia was quickly alleviated. This led to the assumption that in the ashes and ash extracts there was some inorganic substance other than iron that was concerned in hemoglobin regeneration. In the next paper in this series from the University of Wisconsin, Hart, Steenbock, Waddell, and Elvehjem (29) revealed that this other inorganic substance was copper.

Using a liver preparation that had been found effective in the treatment of pernicious anemia in man, they found it to be effective in curing anemia in rats kept on a whole milk diet supplemented with iron. The ash of this preparation was also effective. By extracting the liver preparation with HCl and treating with H_2S and $NH_4OH\text{-}(NH_4)_2S$, the antianemic potency was obtained in a concentrated form in the H_2S fraction. Copper, one of the elements found in the H_2S fraction, proved to be highly active.

At about this same time McHargue, Healy, and Hill (43) also pointed out the importance of copper in hemoglobin formation in the rat.

Several months later Titus and Hughes (58) presented evidence showing that when manganese was added to a milk-iron diet almost, if not quite, as good results were obtained in the building of hemoglobin as when copper was added in the same way. They also obtained a quicker response when copper and manganese were added to a milk-iron diet than when copper alone was used. These workers concluded that a group of substances, rather than a single substance, was active in hemoglobin building.

The favorable results obtained by Beard and Myers (44) through the use of iron alone were explained by Waddell, Steenbock, and Hart (62) as due to contamination with copper. The Wisconsin workers were unable to correct anemia by feeding a pure iron salt, ferric chloride, at as high a level as 10 mg. of iron daily. However, so-called pure iron salts prepared from iron wire did correct anemia when fed at high levels unless freed from traces of copper by treatment with hydrogen sulfide. The analyses of Elvehjem and Lindow (20) likewise showed that many so-called pure iron salts contain copper.

Further proof that the anemia produced on diets of whole milk and iron is due to a deficiency of copper and that copper is a specific supplement to iron in the cure of nutritional anemia was presented by Waddell, Steenbock, Elvehjem, and Hart (61), and by Waddell, Steenbock, and Hart (63). Several liver preparations, hydrogen sulfide fractions of the acid extracts of the ashes of two of them, and copper sulfate, when fed at the same levels of copper intake, served equally well in curing nutritional anemia induced in rats by a whole milk-iron diet. In addition, twelve elements, zinc, chromium, germanium, nickel, cobalt, lead, antimony, tin, cadmium, mercury, arsenic, and manganese were fed separately, but all, with the possible exception of arsenic, were found to be quite inert. Arsenic gave a minimal and temporary response.

The evidence on the specificity of copper as a supplement to iron in preventing or curing nutritional anemia has not been confirmed by all workers in this field. Drabkin and Waggoner (15) succeeded in curing rats made anemic on a whole milk diet by feeding what was claimed to be a copper-free synthetic ration. It has, however, been shown by Elvehjem and Hart (18) that the so-called copper-free synthetic ration of Drabkin and Waggoner was not copper-free, 0.044 mg. of copper being found in 10 grams. Since 0.532 mg. of iron were found in 10 grams of this ration, rats consuming 10 grams a day would be ingesting an optimum amount of iron and copper for hemoglobin regeneration. In a later paper Drabkin and Waggoner (16) presented data showing that, according to their newly modified methods for copper analysis, the amount of copper consumed by the rats on their synthetic low-copper diet was less than that ingested when on an exclusive milk diet. On this basis, according to these workers, "the specificity of copper, or even its necessity, becomes highly questionable."

Myers and Beard (49) reported that, when anemic rats were fed 0.5 mg. of iron plus a trace of manganese, nickel, copper, germanium, or arsenic, definite hemoglobin regeneration resulted. It was pointed out as significant that all these elements, with the exception of nickel, have at some time in the past been recommended therapeutically in the treatment of anemia. Some significance was also attached to the fact that practically all the elements they had found to supplement iron in hemoglobin regeneration stand close to iron in the periodic system. Beard and Myers (4, 5) have worked with several other elements and have shown them to have definite hemoglobin regenerating power, making the following imposing list: iron, copper, nickel, germanium, arsenic, manganese, cobalt, titanium, zinc, rubidium, vanadium, chromium, selenium, and mercury.

Evidence in support of the specific action of copper was recently presented by Lewis, Weichselbaum, and McGhee (35) who, using electrolytically prepared elements, found iron alone ineffective in hemoglobin regeneration. Copper was effective when fed with iron; manganese and cobalt were ineffective. (The manganese used was prepared from C. P. Baker's manganese sulfate).

On the other hand, Keil and Nelson (33) have brought about hemoglobin regeneration in anemic rats through the use of ferric chloride. The combination of iron and copper gave quicker regeneration than iron alone; but the combination of iron and manganese was no better than iron alone.

The entire field has been further obscured by the most recent paper of Drabkin and Miller (14) in which it was reported that anemia induced by a milk diet was relieved by the administration of certain amino acids and their salts.

PRELIMINARY

As the first step in attempting to use the exclusive milk method of feeding for the studies here reported, nothing but milk from three groups of Holstein cows on widely different rations was fed to three respective groups of weanling rats. With two exceptions, both in the same group, all the rats died within 10 weeks. From the appearance of the rats in the period of decline, it was assumed that severe anemia was present. This belief was reported in a paper read before the American Dairy Science Association in June, 1927 (34). (In one group all the rats were dead at the end of 4 weeks. It is believed that some factor other than anemia was responsible for this.)

Since the nature of the cow's ration was thought to be a possible factor in this work, another group of rats was fed nothing but mixed milk from the entire Station herd of Holsteins and Jerseys. Practically the same results were obtained as in the previous trials. It was evident from this that milk from a general source was unable in itself to support growth or prevent death.

Hemoglobin determinations made at this point, August 1927, showed that the blood of rats that had been on an exclusive milk diet for 11 weeks contained between 2 and 3 grams of hemoglobin per 100 cc.; whereas that of normal rats from the stock colony contained from 15 to 16.5 grams per 100 cc. This proved beyond question that a severe anemia existed and suggested that this was a limiting factor in growth studies on exclusive milk diets. It also presented the possibility of obtaining anemic rats quickly without resorting to special breeding on diets low in iron, as suggested by other workers (47, 51).

GENERAL PROCEDURE

Weanling rats 24 to 28 days of age (weighing from 48 to 52 grams) were moved to experimental cages. The mothers from which these weanlings were taken were stock females that had been receiving the regular stock diet of yellow corn meal 67, oilmeal 12, casein 16, alfalfa meal 3, salt 1, calcium carbonate 1, and whole milk *ad libitum*. At first, four rats, two males and two females, were placed in each cage. This was satisfactory when milk was fed *ad libitum* and it was desired to produce anemia or for preliminary work on supplementary feeding, but, when accurate additions to the milk were made, segregation was essential. As the object of this study was to prevent anemia from developing, the substance to be tested was added from the beginning in most cases. Control groups received no addenda, except in some cases after severe anemia had developed when it was desired to test the corrective possibilities of some substance. In such cases the hemoglobin content of the blood was allowed to drop to between 1.38 and 2.07 grams per 100 cc. before adding the substance under consideration. This afforded a very rigid test of hemoglobin-forming power.

After the animals were six weeks of age hemoglobin determinations were made weekly on a different representative of each group. A small piece was cut off the end of the tail with a razor and a freely-flowing drop of blood was transferred by capillarity to the pipette of a Dare hemoglobinometer[2] in which the percentage of hemoglobin was determined. (It was later shown by McKay (44) that no difference existed in the hemoglobin value of peripheral or central blood of the same animal). Three readings were made of each sample, the average being taken for record.

No difficulty was encountered in obtaining a suitable sample of blood from the tail. The rat was held in the bare hands by an assistant while the operator cut the tail, made the blood transfer, and read the hemoglobinometer. It was found unnecessary to apply warm water to the tail to stimulate blood flow. In very stubborn cases gentle stroking of the tail produced the desired result. The blood flow was stopped by applying a solution of silver nitrate and alum powder.

The experimental cages were made of galvanized iron hardware cloth and were provided with screen bottoms. The milk was fed in glass beakers attached to the side of the cage with baling wire, and the amount consumed was estimated as accurately as possible. It was found that, when the appetite was not impaired, the per capita daily consumption of milk at two months of age was about 50 cc. When continued at this level good growth was possible, provided the milk was suitably supplemented.

Milk was fed twice a day, the supplement being added to the afternoon feeding (six times a week), at which time a small enough

[2]It was realized that the Dare hemoglobinometer had been shown to be less accurate than other instruments for determining the absolute amount of hemoglobin, but, since many determinations were to be made and since relative rather than absolute values were considered adequate, this instrument was adopted.

quantity was fed to insure complete consumption. In order to eliminate the factors of breed and environment, the milk used was from Holstein cows kept under winter feeding conditions constantly and receiving a normal dairy ration of alfalfa hay, corn silage, corn, oats, bran, and oilmeal.

SYMPTOMS OF NUTRITIONAL ANEMIA

When rats are first placed on an exclusive whole milk diet, growth proceeds at a rapid rate for several weeks, even though weekly hemoglobin determinations show a decline in hemoglobin and the exposed skin parts and eyes appear paler. Growth slows up after about 4 weeks and then declines, following, in general, the food consumption. Males develop priapism. Rats suffering from severe nutritional anemia are very inactive and weak. Their hair is straggly and is chalk-white in color. The eyes are whitish-yellow, and all the exposed skin surfaces are colorless. Figure 1 shows a rat suffering from severe anemia.

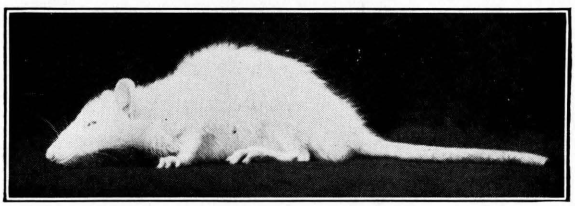

Fig. 1.—Rat suffering from severe nutritional anemia

Post-mortem examination confirms at first glance the external symptoms of anemia. All the tissues of reddish color under normal conditions are much lighter. The heart, liver, and spleen are usually enlarged. The liver and spleen vary in color from yellow to almost black, and at times are speckled. In many cases evidence of intestinal hemorrhage and impaction is found. The testes of males as a rule are very small. Most of the symptoms described here were similar to those reported by Brouwer (7), who fed exclusive milk diets to rabbits.

In order to establish definitely the relative size of certain organs of anemic rats and those of normal individuals, organs from rats that had died while on an exclusive milk diet were weighed. The weights obtained are compared in Table 1 with the normal weights found by Donaldson (12). Enlargement of the heart has been observed to accompany anemia by Forman and Daniels also (22).

TABLE 1.—Weight of Various Body Organs of Anemic Rats Compared with Normal Weights, According to Donaldson

(Weight per 100 grams of body weight)

	Males		Females	
	Anemic	Normal	Anemic	Normal
	Gm.	*Gm.*	*Gm.*	*Gm.*
Heart	0.985	0.470	1.137	0.473
Lungs	1.616	0.665	1.162	0.670
Liver	6.439	6.000	6.391	6.040
Spleen	0.307	0.278	0.539	0.281
Kidneys	1.527	0.944	1.630	0.951

Owing to the fact that the increase in the size of the testes plotted against increase in body weight does not give a linear function curve, as is practically the case with the other organs listed in Table 1, the weights of testes from anemic individuals are compared with the weights of the testes of normal individuals of the same age (Table 2). It will be noted that a few anemic individuals had larger testes than did the normals, but, if other weights taken from individuals suffering from anemia but fed some supplement not affecting anemia are considered, the statement holds true that the testes from anemic rats are smaller, the difference in size becoming greater as the age increases.

TABLE 2.—Weights of Testes of Anemic Rats Compared with Normal Weights, According to Donaldson

Age	Body weight		Weight of testes	
	Anemic	Normal	Anemic	Normal
Days	*Gm.*	*Gm.*	*Gm.*	*Gm.*
93	68	155.3	0.50	1.854
64	79	89.6	0.80	1.173
115	79	185.2	0.60	2.073
76	100	121.1	2.00	1.541
98	84	162.9	0.50	1.920
63	68	87.2	1.23	1.125
61	86	82.5	1.70	1.055

Figure 2 illustrates the relationships shown by the figures in Tables 1 and 2.

In Figure 3 are presented curves showing the rate of growth and hemoglobin values of rats placed upon an exclusive whole milk diet at weaning age. These curves are typical of a great many that have been obtained on rats fed in this way and agree closely with the results obtained by Waddell, Steenbock, Elvehjem, and Hart (31). The techniques described here and in the publication just cited were worked out independently.

Hemoglobin determinations made on weanling rats gave values ranging from 10.33 to 11.02 grams per 100 cc., showing that at this stage the animals were not anemic. However, in rats allowed to continue on the stock diet, the hemoglobin value rises in a short time to from 15.15 to 16.02 grams per 100 cc.

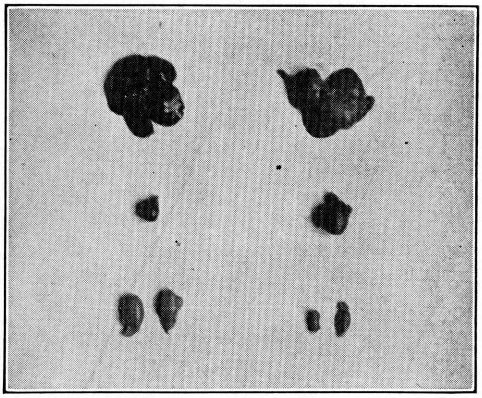

Fig. 2.—Showing the relative size of the liver, heart, and testes of a normal and an anemic rat. Left—normal; right—anemic.

The large heart and small testes are typical.

As indicated in Figure 3, the size of the animal when placed on the exclusive milk diet influenced the subsequent degree of growth and the rate at which the hemoglobin decreased. This has been found to be true generally. It is believed that when dealing with closely inbred stock the food consumption is as important as heredity in determining size. Hence, in the case of weanling rats of the same age, the larger ones have a greater iron reserve. To insure the development of severe anemia in a short time, rats should be transferred to the whole milk diet as soon as possible after they have reached the stage when they start to consume some of the mother's diet. It is suggested that the mother be placed on an exclusive milk diet at the time the young rats become able to eat independently. This would prevent the intake of materials other than those found in the mother's milk and might prevent some of the contradictory results obtained in different laboratories using different stock diets and different techniques with respect to weaning the young.

EXPERIMENT I. THE SUPPLEMENTARY VALUE OF INORGANIC IRON AND COPPER

Since the exclusive milk method of feeding could not be used for comparing the food value of milk from different sources, it became necessary to search for a supplement that would prevent the development of anemia. Such a substance would need to be sufficiently potent to insure protection through the addition of a very small quantity, particularly if a food substance. If a chemical, it could not be of such a nature as to bring about a reaction that would alter the original form of the milk. Owing to the great emphasis that had been placed upon iron in anemia in the past, it was natural to turn first of all to some source of iron as the necessary supplement.

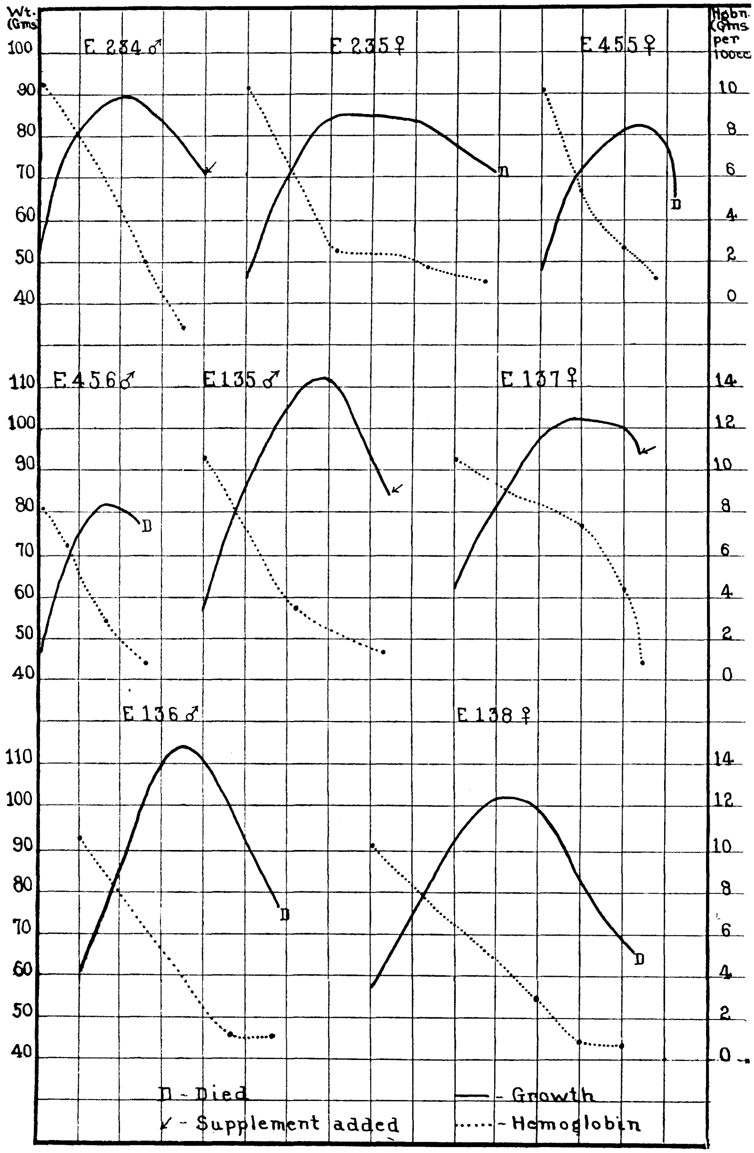

Fig. 3.—Showing growth curves and hemoglobin values on an exclusive whole milk diet

Inorganic sources of iron were chosen because of the work of Hart, Elvehjem, Waddell, and Herrin (27), which showed that the ash of lettuce or cabbage corrected anemia, and of Mitchell and Schmidt (47), which showed considerable success through the use of certain inorganic iron salts.

The technique employed was essentially the same as that described under *Preliminary*. However, instead of applying silver nitrate and alum powder to the tail after cutting, a hot spatula was applied momentarily. This proved to be much more satisfactory as it reduced the loss of blood to a minimum. It was found that even weanling rats were unaffected by this treatment. Hence, this technique was employed in all the succeeding experiments.

While it was found by Elvehjem, Herrin, and Hart (19) that the cow's feed did not influence the iron content of milk, the precaution was taken to use milk from the same cows throughout. Milk from three cows fed a normal ration and not given access to pasture was saved each day and combined for feeding.

Samples of milk from each cow were analyzed from time to time for iron and copper. The method of Elvehjem and Hart (17) was used for iron, and the Biazzo method (6) for copper. Both the iron and the copper content of the milk from each cow varied. The average iron content of the milk was 0.00024 per cent, and the average copper content 0.00005 per cent.

EXPERIMENTAL

As an iron supplement, ferrous sulfate (Baker's C. P.) was first tried. A solution of the salt was made so that 1 cc. contained 0.4 mg. of iron. This quantity was considered by Abderhalden (1) to be sufficient for the requirements of the rat. Mitchell and Schmidt (47) found that when 0.4 mg. of iron from an easily available source was added to the diet of anemic rats a rapid rise in hemoglobin occurred. Consequently, 1 cc. of iron solution was incorporated in the milk fed each rat. The iron addition was made as soon as the rats were placed on experiment. It was added to a sufficiently small amount of milk in the afternoon to insure complete consumption. Additional milk was then fed in the morning.

A solution of ferric citrate (Baker's C. P.) was prepared and fed in a similar manner to that employed with the ferrous sulfate.

It was thought that the state of iron might influence its availability; hence, a colloidal solution of ferric oxide was prepared and fed at the same level and in the same manner as were the other iron additions. As a further check on this point, a colloidal solution of ferric oxide was compared with a true ferric chloride solution of equal iron content. In order to insure an adequate iron intake, 0.8 mg. of iron, twice the amount found by others to meet the requirements, was fed to each rat.

About this time the information contained in the article by Hart and associates (29) was made public. Consequently, copper sulfate was tried, together with iron, to overcome the anemia. Copper sulfate alone was fed to another group of rats, and a group of controls on milk only were maintained at the same time.

RESULTS

The growth curves of the rats receiving the ferrous sulfate addition, together with curves showing the trend of the hemoglobin content of the blood, are given in Figure 4. Rat E 147 developed severe respiratory trouble shortly after the first hemoglobin determination was made and was in such poor condition that no further data as to hemoglobin were obtained. The only effect of the ferrous sulfate was to reduce slightly the rate at which the hemoglobin content of the blood decreased. It may be of significance that one rat in this group died of respiratory trouble and another, E 149, had puffy eyes during the last few weeks of the trial.

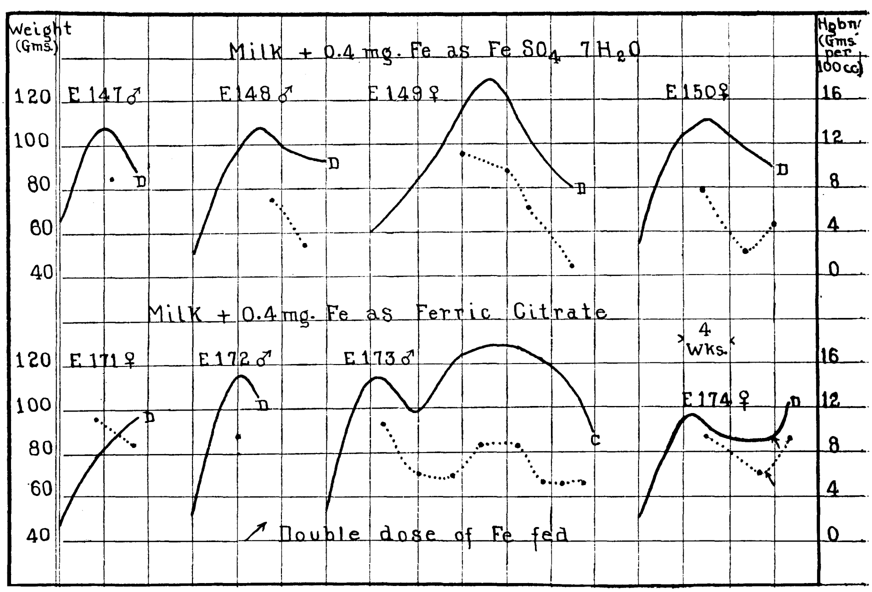

Fig. 4.—The addition of ferrous sulfate or ferric citrate to an exclusive milk diet did not prevent anemia

Similar results were obtained with the ferric citrate. Respiratory trouble and eye trouble prevailed in this group. The conjunctiva became red and swollen and the lids were sticky. This condition closely resembled xerophthalmia due to a vitamin-A deficiency. It might appear that both ferrous sulfate and ferric citrate tend to induce the "salt ophthalmia" described by McCollum, Simmonds, and Becker (39, 40), and explained by Jones (32) as due to destruction of vitamin A through oxidation. Our results are not in accord with those of McCollum and associates, who found that when ferrous sulfate was replaced by ferric citrate the ophthalmia disappeared. Furthermore, ulceration and perforation of the eyeballs have been observed in this laboratory in rats on an exclusive milk diet, with no iron addition.

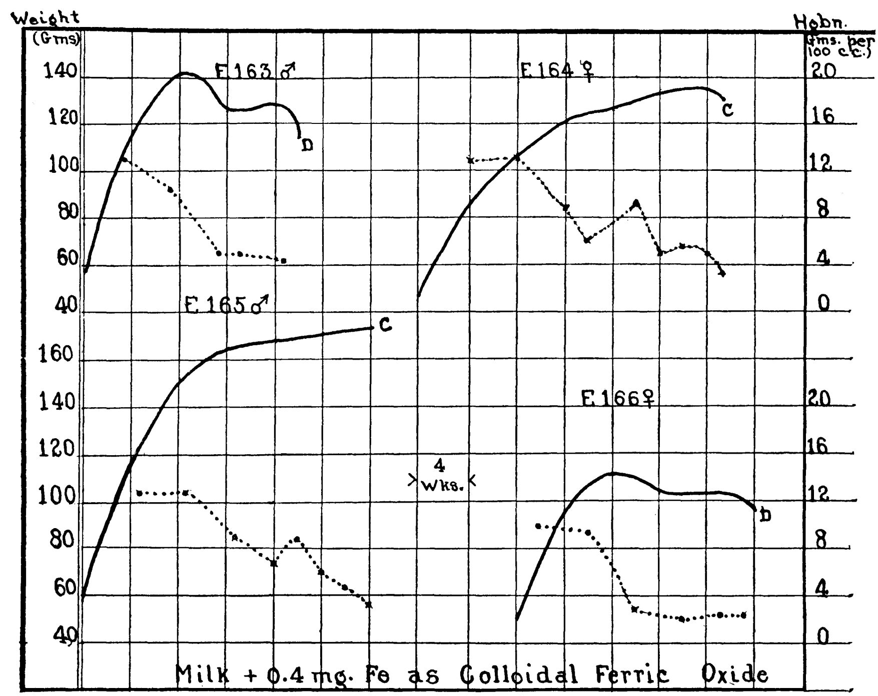

Fig. 5.—The addition of colloidal ferric oxide prolonged life somewhat but did not prevent anemia

It is apparent from Figure 5 that the colloidal nature of the iron did not enable it to prevent anemia from developing. However, life was prolonged considerably and the rats were in better condition than those receiving either ferrous sulfate or ferric citrate. When compared with ferric chloride, however, no advantage was shown for colloidal ferric oxide (Figure 6). This trial demonstrated that, even with such a high iron intake, the hemoglobin content of the blood decreased rather rapidly. This observation was confirmed by Waddell, Steenbock, and Hart (62) and indicated that some factor other than iron is necessary for hemoglobin formation in the rat.

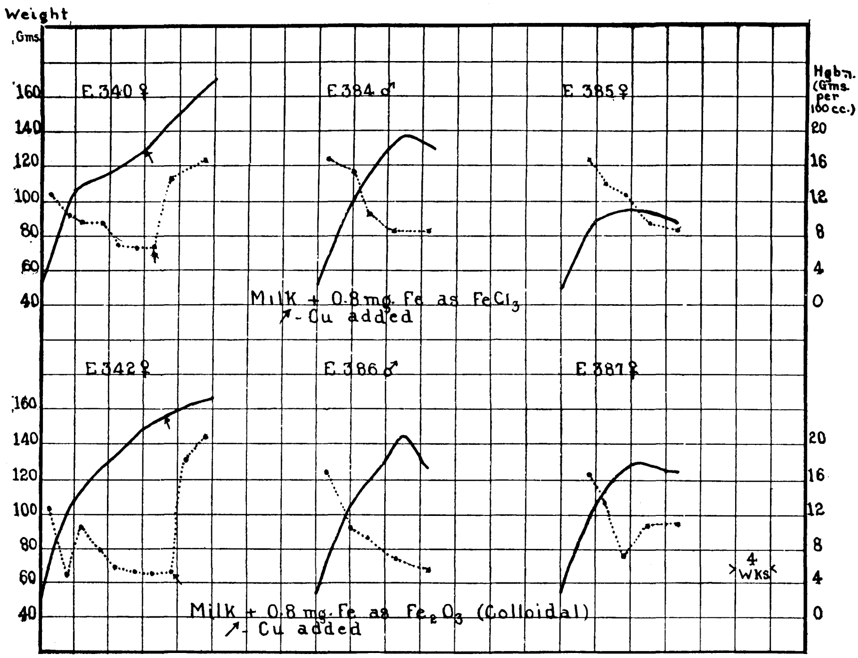

Fig. 6.—Colloidal ferric oxide did not prove superior to ferric chloride in preventing anemia. The high iron intake also did not prove beneficial

That this factor is copper, as claimed by the Wisconsin workers, was confirmed by the results obtained when 0.16 mg. of copper was added daily to the milk fed to rats receiving 0.2 mg. of iron, and when 0.2 mg. of iron and 0.16 mg. of copper or 0.4 mg. of iron and 0.32 mg. of copper were fed simultaneously from the beginning (Figures 6, 7, and 8).

After growth had ceased and hemoglobin was at a low level, the addition of 0.16 mg. of copper to the milk-iron diet resulted in an immediate response in both body weight and the amount of hemoglobin.

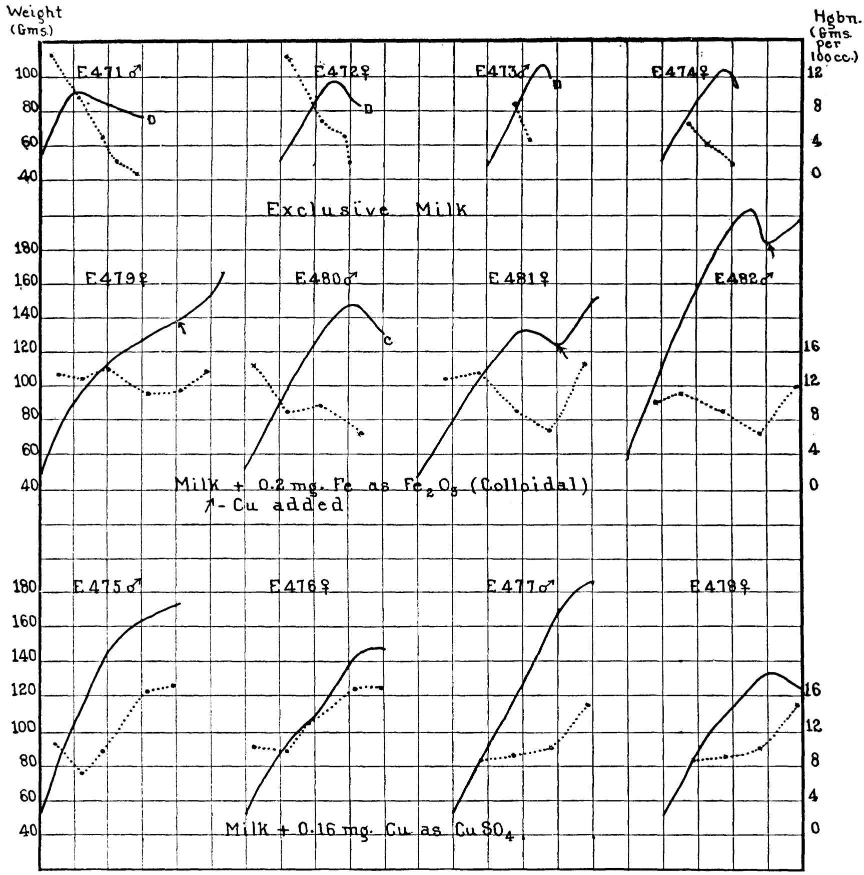

Fig. 7.—The addition of copper as copper sulfate after anemia had developed on a milk-iron diet resulted in an immediate favorable response. The addition of copper alone from the beginning prevented anemia

The addition of copper alone (Figure 7) allowed good growth and caused the hemoglobin content of the blood to rise slowly to the normal level. Repetitions of this particular type of feeding have given results not as striking. However, the addition of copper alone has always stimulated hemoglobin production in anemic rats, after which the hemoglobin is maintained at a constant, rather low level, for a considerable length of time. This cannot be explained on the basis of the iron impurity in C. P. anhydrous copper sulfate as this amounted to only 0.000008 mg. daily. Since the amount of iron furnished by the milk was only 0.10 to 0.20 mg., it appears that either there is a requirement for a very small amount of copper for hemoglobin formation, or the iron present in the milk is made more available through the presence of copper. If the latter view is correct, it follows that the iron requirement of the rat is not as great as was formerly supposed. If the copper exerts only a catalytic effect, the amount present in the milk consumed daily, amounting to 0.025 mg. (based on an average consumption of 50 cc. of milk containing 0.5 mg. of copper per liter), should exert some effect on the availability of the iron in the milk. That more copper than that present in the milk is necessary indicates that copper might perform some function other than one catalytic in nature.

The effect of adding copper to the milk and iron diet of anemic rats is shown by the curves for rats E 481 and E 482 (Fig. 7) and rats E 340 and E 342 (Fig. 6). This confirms the historic case described by Hart and associates and has been observed repeatedly in this laboratory without record of a single failure.

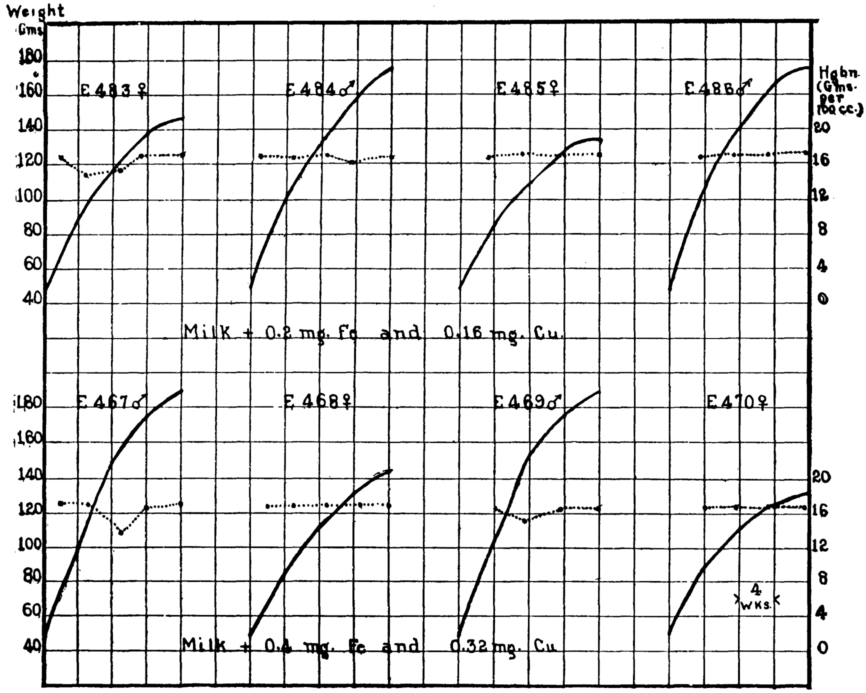

Fig. 8.—The addition of 0.2 mg. of iron and 0.16 mg. of copper, or 0.4 mg. of iron and 0.32 mg. of copper, allowed the hemoglobin to stay at a normal level

Figure 8 offers further evidence of the supplementary effect of copper and iron additions to milk and shows that the daily iron requirement of the rat is certainly no greater than 0.4 mg. Just as good results were obtained with 0.2 mg. as with 0.4 mg. additions of iron.

The remarkable response obtained by the addition of both copper and iron is apparent from Figure 9.

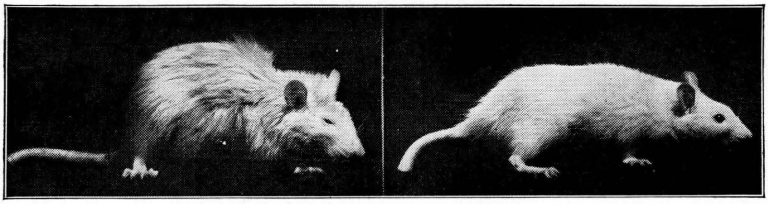

Fig. 9.—Left—rat fed on exclusive whole milk diet; hemoglobin, 10 per cent of normal. Right—the same rat after iron and copper additions daily for two months; hemoglobin, 100 or normal

CUSSION

It has long been recognized that the most serious dietary deficiency of milk is its low iron content, but there is apparently an equally serious deficiency of copper. The natural way to overcome these deficiencies would seem to be to increase the copper and iron content of milk by feeding larger quantities of these elements than are found in the usual ration. However, it has been shown by Elvehjem, Herrin, and Hart (19) and by Elvehjem, Steenbock, and Hart (21) that the iron and copper content of milk cannot be increased by feeding iron and copper salts to the cow.

It is also apparent that for hemoglobin formation in the rat the availability of the iron is more important than the amount present, and that the iron is made more available through the addition of copper.

The addition of small amounts of iron and copper to an exclusive milk diet should make it possible to use such a system of feeding for determining the total nutritive effect of milk produced under different conditions.

EXPERIMENT II. THE SUPPLEMENTARY VALUE OF VARIOUS CONSTITUENTS OF SYNTHETIC BASAL RATIONS

The nutritional disaster encountered when rats received an exclusive whole milk diet raised the question, "Is milk deficient in one of the basic constituents of a balanced diet?" Rats had been successfully raised on synthetic rations; yet milk, ordinarily considered a complete food, could not accomplish this. Some clue as to the deficiency of milk responsible for this failure could be obtained by supplementing the milk diet with each of the constituents of an ordinary synthetic diet. With this in mind, the following experiment was undertaken.

EXPERIMENTAL

Albino and piebald rats were fed a basal ration of liquid whole milk to which was added one of the constituents of ordinary synthetic basal rations, such as casein, agar, starch, yeast, salts, cod-liver oil, wheat germ oil, gelatin, and rice polishings. This procedure resembles somewhat that followed by Mattill and Conklin (37), but these investigators were concerned primarily with reproduction. Since vitamin D is often imparted to a basal ration by means of irradiation, one group of rats was fed milk irradiated in a thin layer under a Cooper-Hewitt lamp for 10 minutes at a distance of 14 inches from the burner.

Two male and two female rats were placed together in a cage in order to allow every opportunity for reproduction to occur. The amount of each supplement fed, arbitrarily chosen, was weighed or measured each day and added to the milk. This was thoroughly stirred in glass beakers and placed in the cages in the afternoon. The amount of milk fed at this time was small enough to insure complete consumption. In the morning more milk was added to the beakers, so that the consumption of milk was *ad libitum*. Weights were recorded weekly and hemoglobin determinations were made on one rat of each group each week. The technique of obtaining blood samples and of determining hemoglobin was the same as that described under Experiment I.

RESULTS

The results, based upon body weights and hemoglobin values after two and three months on the respective diets, are presented in Table 3.

It is very evident that yeast, McCollum's salt mixture 185, agar, casein, and starch were helpful in preventing anemia. Of these substances, yeast was by far the most effective, the hemoglobin of the rats receiving it remaining at a high level throughout. In the case of the other supplements there was a drop in hemoglobin during the critical period, but as the rats matured there was gradual regeneration.

TABLE 3.—Gain in Weight and Hemoglobin Content of the Blood of Rats Fed Milk Only and Milk Plus Various Supplements

Rat No.	Initial weight	After 2 months		After 3 months	
		Weight	Hemoglobin	Weight	Hemoglobin
Milk exclusively (ad lib.)					
	gm.	*gm.*	*gm. per 100 cc.*	*gm.*	*gm. per 100 cc.*
E135♂	59	90	1.38—	*	*
E136♂	60	95	1.38—	dead	....
E137♀	64	95	1.38—	*	*
E138♀	58	96	2.34	dead	....
Milk plus Dried Yeast (0.4 gm. per rat)					
E139♂	61	155	16.52—	211	16.52
E140♂	62	183	15.84	233	15.84
E141♀	62	123	16.52	152	16.52
E142♀	60	148	13.77	172	13.77
Milk plus Cod-liver Oil (3 drops per rat)					
E143♂	63	137	2.07	dead	....
E144♂	61	dead	....	....	....
E145♀	61	85	2.75	dead	....
E146♀	56	86	2.62	dead	....
Milk plus McCollum's Salt Mixture 185 (0.25 gm. per rat)					
E155♂	60	93	8.68	134	13.08
E156♂	61	152	12.39	177	11.70
E157♀	56	117	16.52	126	16.52
E158♀	62	117	12.39	137	12.67
Milk plus Gelatin (0.25 gm. per rat)					
E167♂	52	150	2.75	169	2.24
E170♂	54	130	2.34	dead	....
E168♀	52	138	3.86	150	3.86
E169♀	49	dead	....	dead	....
Milk plus Agar (0.50 gm. per rat)					
E175♂	52	180	9.36	214	7.57
E178♂	52	160	6.75	170	9.36
E176♀	50	133	8.12	135	4.96
E177♀	49	128	7.16	135	10.33
Milk plus Casein (2 gm. per rat)					
E180♂	64	174	13.08	190	15.15
E182♂	52	195	13.77	205	11.15
E179♀	59	134	13.77	145	16.25
E181♀	49	138	15.15	died from loss of blood	
Milk plus Starch (2 gm. per rat)					
E185♂	51	175	8.26	218	10.60
E186♂	49	157	7.30	190	11.02
E183♀	50	130	8.40	158	10.88
E184♀	49	135	7.57	158	13.77
Milk plus Rice Polishings (0.4 gm. per rat)					
E247♂	51	144	3.17	168	2.62
E249♂	54	146	2.34	150	1.79
E246♀	51	117	5.23	129	5.92
E248♀	53	dead	....	....	....

*Yeast added.

TABLE 3.—Gain in Weight and Hemoglobin Content of the Blood of Rats Fed Milk Only and Milk Plus Various Supplements—Continued

Rat No.	Initial weight	After 2 months		After 3 months	
		Weight	Hemoglobin	Weight	Hemoglobin
Milk plus Wheat Germ Oil (3 drops per rat)					
E251♂	62	dead	....	....	....
E253♂	58	88	1.38	dead	....
F250♀	55	65	3.03	dead	....
E252♀	48	dead	....	....	....
Milk, Irradiated (ad lib.)					
E238♂	52	72	1.38	dead	....
E240♂	50	71*	1.38*	*	*
E239♀	52	89	2.48	dead	....
E241♀	47	106*	1.38*	*	*

*Yeast added.

In order to find a possible explanation of the behavior of these substances in preventing anemia, determinations were made of their copper and iron content. Copper was determined by the Biazzo method (6) and iron by the method of Fowweather (23), except in the case of the salt mixture where the Elvehjem and Lindow method for copper (20) and the Elvehjem and Hart method for iron (17) were used. The results of these analyses are presented in Table 4.

TABLE 4.—Iron and Copper Content of Supplements, and Daily Intake

Material	Iron	Copper	Daily Intake	
			Fe	Cu
	Pct.	*Pct.*	*mg.*	*mg.*
Yeast	0.0918	0.0032	0.49	0.04
Salts 185	0.2561		0.76	0.03
Agar	0.0175		0.21	0.03
Casein	0.0162	0.0024	0.44	0.07
Starch	0.0135	0.0073	0.39	0.04

The data shown in Table 4 indicate a rather definite relationship between the amounts of copper and iron required by the rat for normal hemoglobin formation. Wherever at least 0.45 mg. of iron and 0.04 mg. of copper were furnished the hemoglobin level was highest, as in the case of yeast and casein. Hart, Steenbock, Waddell, and Elvehjem (29) secured excellent results through the administration six times per week of 0.05 mg. of copper and 0.5 mg. of iron.

DISCUSSION

The exceptional response obtained through supplementing milk with 0.4 gram of yeast daily may be attributed to an adequate intake of copper and iron. The amounts of copper and iron supplied were probably optimum, the ratio between these two elements being 1:12.25. That no organic factor was involved was shown when the ash of 0.4 gram of yeast was used with equally good results. Evidently vitamin B was no factor, as negative results were obtained with rice polishings. The same applies to vitamins A, D, and E, as cod-liver oil, irradiated milk, and wheat germ oil likewise exerted no favorable influence. Cartland and Koch (9) concluded that vitamins A, B, and E probably were not essential for hemoglobin formation in the rat. These same investigators found, as did McKay (44), that casein promoted hemoglobin synthesis.

The body weights at two and three months (Table 3) indicate that, when rats are given whole milk *ad libitum*, the only deficiency exists in the mineral portion of the diet. When additional protein and energy in the form of casein and starch, respectively, were added to the basal milk diet, good growth occurred. The inferior growth of rats receiving the salt mixture, however, points to a mineral deficiency. The rats in this group received an adequate supply of iron as iron citrate, but their intake of copper was inadequate. The addition of bulk in the form of agar had no effect on the rate of growth and was not very effective in preventing anemia because at the level fed the total intake of copper and iron was insufficient.

Yeast, casein, agar, starch, and McCollum's salt mixture 185 are often used in synthetic basal rations. When iron metabolism or hemoglobin regeneration studies are conducted and a basal ration containing any of the above ingredients is contemplated, a careful evaluation of both the copper and iron content of the diet should be made.

EXPERIMENT III. THE SUPPLEMENTARY VALUE OF YEAST

In the preceding experiment it was found that when 0.4 gram of yeast from our stock supply was added to an exclusive milk diet the rats thrived and their hemoglobin remained at a high level. This could not be attributed to vitamin B (complex), as it had been previously shown that 16 cc. of milk were adequate when fed in addition to a basal ration free from this factor and the rats in this experiment were consuming at least twice that amount. Furthermore, rice polishings, supplying the B complex, were not beneficial. Also, the ash of the same yeast was equally effective, suggesting that the factor or factors involved were inorganic in nature.

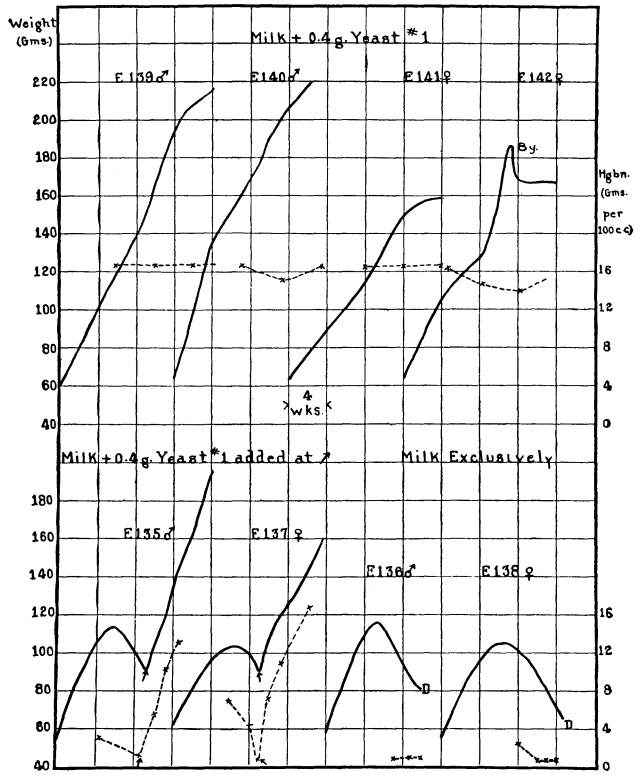

Fig. 10.—Showing the antianemic potency of stock supply of yeast (#1) as a curative and as a prophylactic. This sample was rich in iron (0.0923 per cent) and contained a fair amount of copper.

EXPERIMENTAL

Since iron had always been associated with hemoglobin, an analysis of the yeast for iron was made. The publication of Hart, Steenbock, Waddell, and Elvehjem (29) at this time suggested copper as a factor in hemoglobin formation. Hence, analysis of the yeast for this element was also made. The sample was found to contain 0.0923 per cent iron and 0.0032 per cent copper. This meant that the daily intake from 0.4 gram of yeast was 0.3692 mg. of iron and 0.0128 mg. of copper. Figure 10 shows the effect of the addition of 0.4 gram of stock dried yeast to an exclusive milk diet, both as a prophylactic and as a curative substance. The curves of two representative rats not receiving the supplement are given for comparison.

The stock supply of yeast was a dehydrated product consisting of pure starch-free yeast cells. This supply having become exhausted, a new one was obtained. When 0.4 gram of this new supply was used to supplement milk the results were quite different from those obtained with the original supply. Growth was excellent, but the hemoglobin level became lower and lower until the rats receiving it were anemic. Analysis showed that this second sample of yeast contained 0.0178 per cent iron and 0.0035 per cent copper, considerably less iron than in the first sample and about the same amount of copper. Figure 11 shows the difference in the effect of these two samples of yeast on the hemoglobin of representative rats, both sets of curves being compared with the curves of rats receiving an equal weight of dried hog liver.

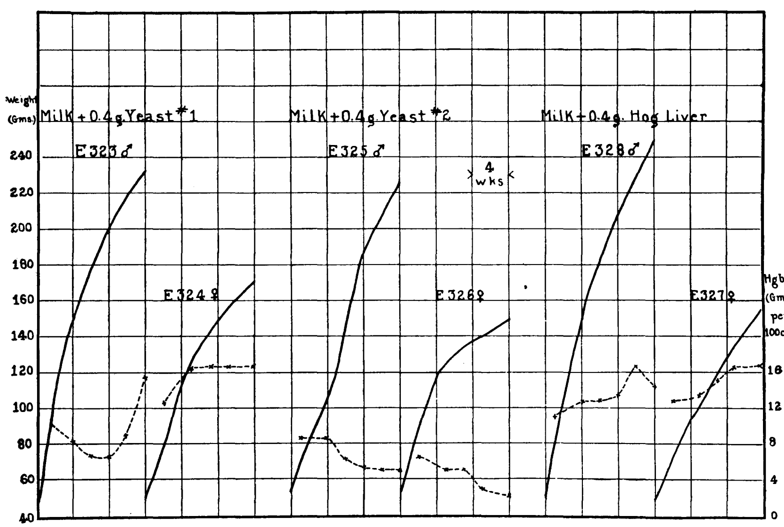

Fig. 11.—Showing the difference in antianemic potency between stock supply of yeast (#1) and of a new supply of yeast from the same source (#2). The chief difference in chemical composition existed in the iron content.

A third sample of yeast from the same source gave still different results, these lying between those of the first two samples. It was then decided that before any conclusion as to the value of yeasts in general as an antianemic substance could be made samples from various sources would need to be assayed. Consequently, 10 samples of yeast were obtained from six of the leading manufacturers of yeast, and their antianemic potency was determined.

Most of the samples were obtained in the dry form. One sample was delivered to the laboratory in fresh cakes, and another arrived in the form of a paste. These latter two samples were dried and pulverized. Each sample was analyzed for dry matter, total nitrogen, ash, copper, and iron. The Official Methods were used for dry matter, total nitrogen, and ash. The Biazzo method (6) was used for copper, and the method of Fowweather (23) for iron.

Each sample was tested as a prophylactic and as a curative. In the prophylactic assay, 0.4 gram of dried sample was added daily to the milk from the beginning of experimental feeding. The feeding period lasted for 16 weeks. In the curative assay the hemoglobin of the rats was allowed to drop to from 2 to 4 grams per 100 cc. of blood, at which time 0.4 gram of yeast was added daily. The fortified ration was continued until 16 weeks had elapsed from the beginning of the experiment. Hemoglobin determinations were made every two weeks as a rule. In some cases, where a critical condition seemed imminent, hemoglobin determinations were made oftener.

TABLE 5.—Analysis of Yeasts (per cent)

Number	Dry Matter	Total Nitrogen	Ash	Copper	Iron
1	97.04	7.525	12.550	0.0032	0.0923
2	95.78	7.410	9.975	0.0035	0.0178
3	95.92	8.335	11.880	0.0041	0.0281
4	92.81	8.730	6.980	0.0045	0.0153
5	90.09	8.200	11.790	0.0022	0.0193
6	95.78	8.085	6.540	0.0038	0.0186
7	90.42	4.215	6.130	0.0056	0.0154
8	88.53	6.870	8.805	0.0034	0.0194
9	92.26	6.870	7.360	0.0026	0.0086
10	92.43	9.310	6.160	0.0046	0.0142

RESULTS

The antianemic potency of the various samples of yeast is shown graphically in Figures 12 to 15. With two exceptions the yeasts showed some potency, the degree of effectiveness varying considerably. This was taken into consideration in giving a final rating to each sample. Yeasts 3 and 7 were particularly effective; yeast 5 was practically without effect; while yeast 8 exhibited very weak hemoglobin regenerating power. Table 5 shows that yeasts

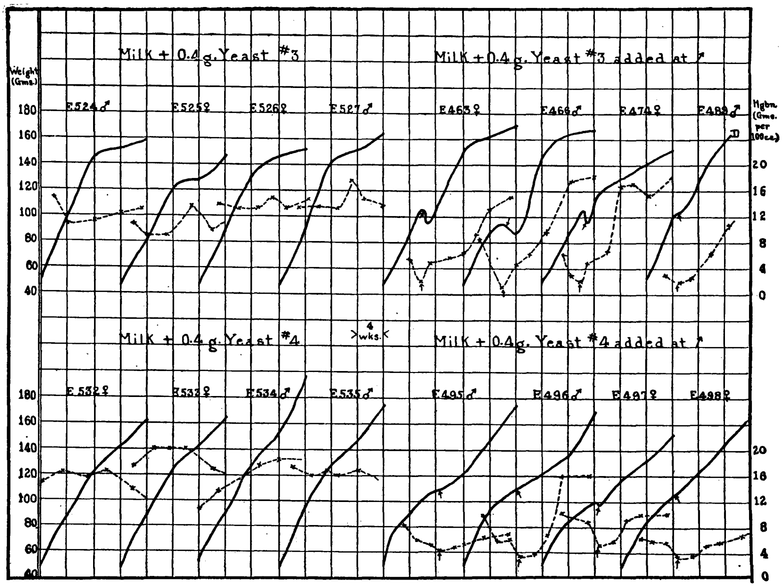

Fig. 12.—Yeast 3 came from the same source as yeasts 1 and 2. It contained more iron than yeast 2 and more copper than either 1 or 2

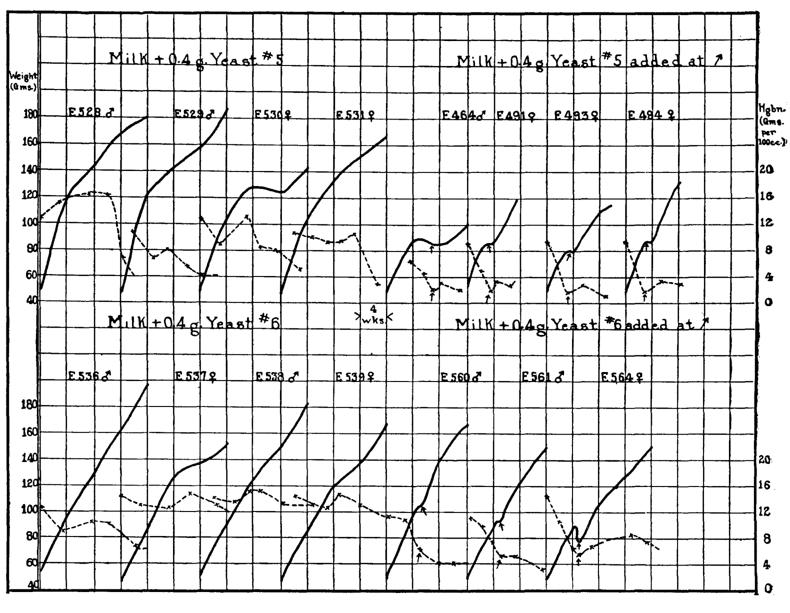

Fig. 13.—Yeasts 5 and 6 came from the same company but were prepared in different ways. No. 5 contained less copper than any other sample and gave the poorest results. No. 6 was grown in molasses wort, with ammonium salts.

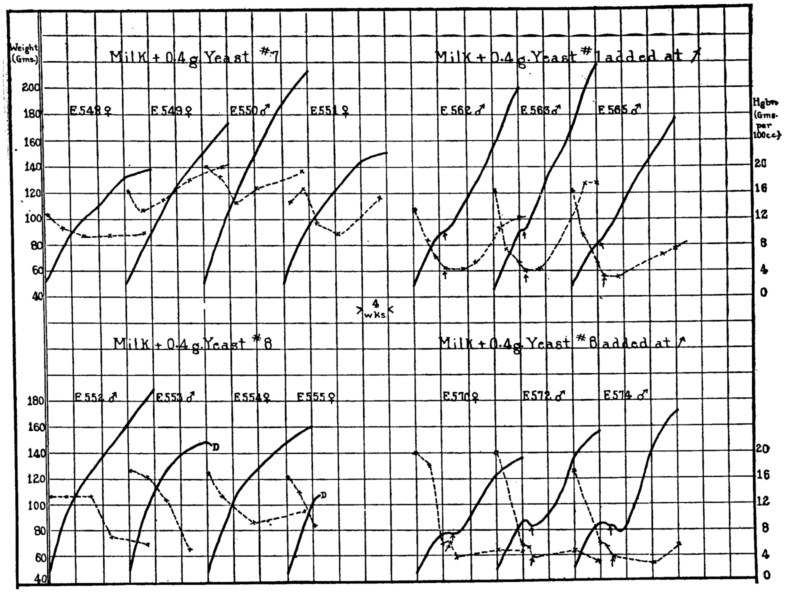

Fig. 14.—Yeasts 7 and 8 came from the same company but were prepared in different ways. No. 7 was grown on grain extract; while No. 8 was grown on molasses. No. 7 contained more copper than any other sample.

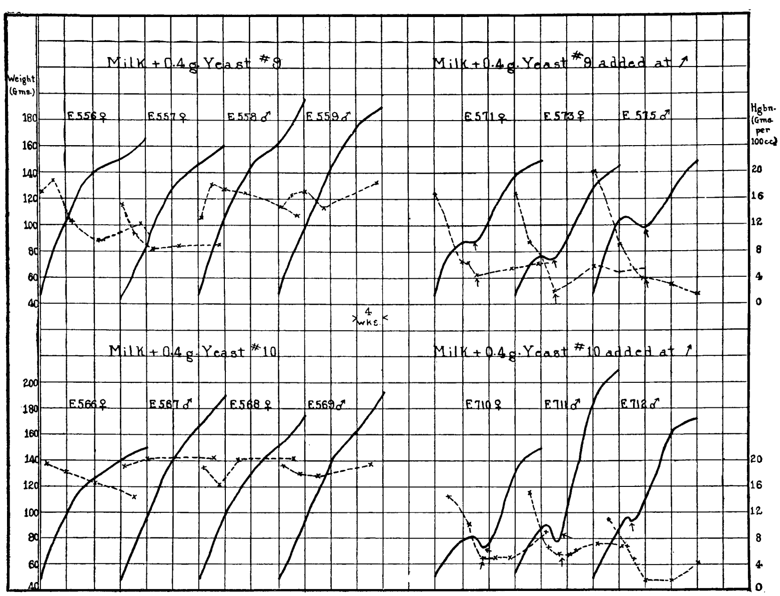

Fig. 15.—Yeast 9 was low in both iron and copper. Yeast 10 was low in iron. These samples were much more potent as prophylactics than as curatives

3 and 7 were rich in copper, that yeast 5 contained the least copper of any sample, and that yeast 8 contained fair amounts of both copper and iron.

TABLE 6.—Relation Between Copper Content and Antianemic Potency

Number	Per cent Copper	Antianemic Potency
7	0.0056	Very good
10	0.0046	Good
4	0.0045	Good
3	0.0041	Very good
6	0.0038	Fair
2	0.0035	Fair
8	0.0034	Very Poor
1	0.0032	Excellent
9	0.0026	Fair
5	0.0022	Very poor

In order to see if any relationship could be shown to exist between the copper and iron content of the yeasts and their antianemic potency, the various samples are listed in Table 6 in descending order according to their percentage copper content and classified according to their effectiveness as shown in the charts. In Table 7 they are treated similarly according to their percentage iron content. With one notable exception the samples at the top of the list in Table 6 are those that showed marked effectiveness. In Table 7 no relationship between iron content and antianemic potency is apparent.

TABLE 7.—Relation Between Iron Content and Antianemic Potency

Number	Per cent Iron	Antianemic potency
1	0.0923	Excellent
3	0.0281	Very good
8	0.0194	Very poor
5	0.0193	Very poor
6	0.0186	Fair
2	0.0178	Poor
7	0.0154	Very good
4	0.0153	Good
10	0.0142	Good
9	0.0086	Fair

DISCUSSION

The results obtained in this study show that yeasts vary considerably as to chemical composition. This may be due to the nature of the media, the vessel in which the yeasts are grown, and the materials with which they were in contact during subsequent treatment.

It is likewise shown that yeasts from different sources vary considerably as to antianemic potency. In trying to link this up with their variation in chemical composition it becomes strongly indicative that it is rather closely related to their copper content. The superior value of yeasts 1 and 3 might have been due to the high level of iron intake resulting from their administration. If the theory of the catalytic action of copper on iron is upheld, this would seem to be a satisfactory explanation, as it could be argued that the lower levels of iron intake had not reached the saturation point. On the other hand, yeast 7 furnishes a relatively small amount of iron but was richest of all the samples in copper and showed strong hemoglobin regenerating power. This would indicate a direct utilization of the copper. However, in a complex substance such as yeast, there are other inorganic substances which were not determined and which might have exerted an influence. Titus, Cave, and Hughes (57) have obtained excellent results in hemoglobin regeneration through the use of manganese; while Myers and Beard (49) have obtained responses through the use of a number of different elements.

In view of the work of Waddell, Steenbock, Elvehjem, and Hart (61) and of McHargue, Healy, and Hill (43), together with the results obtained in Experiment I here reported, the part played by copper, as shown in Table 6, is outstanding.

Sure and Kik (53) reported beneficial effects in hematopoietic function through the use of yeast. They found a greater hemoglobin concentration in nursing young when the mothers received a diet containing 10 per cent of dried yeast than when they received the stock diet. Sure, Kik, and Walker (55), referring to some of their own unpublished data, stated that during gestation they found no anemia on synthetic diets containing an abundance of wheat embryo or yeast as sources of the vitamin-B complex. In a later publication Sure, Kik, and Walker (56) concluded as follows: "The character of our results certainly does not show any relation between a deficiency of the vitamin B complex or uncomplicated vitamin B deficiency and pernicious anemia, or, as a matter of fact, does not establish the association with any definite form of anemia." However, upon working with the separate factors in the B complex, Sure, Kik, and Smith (54) found that an anemia occurred when there was a vitamin-G deficiency, accompanied by skin lesions comparable to those found in human pellagra. This was not attributed to a mineral deficiency since no response was obtained by feeding the ash of yeast. This would not enter into anemia produced on an exclusive milk diet since it has been shown by Hunt and Krauss (31) that milk is a good source of vitamin G. It would seem, then, that the hemoglobin-regenerating power shown by certain samples of yeast is in no way related to their richness in the factors of the vitamin-B complex.

That the addition of yeast to an exclusive milk diet may exert some benefit other than in hemoglobin regeneration is indicated in the charts where it is shown that growth continues even after the hemoglobin has reached a low level. This may be due to greater food consumption and to an adequate intake of vitamin B. On an exclusive milk diet there is a decline in growth after the first few weeks. Milk consumption decreases and, since milk has been shown to be a poor source of vitamin B (31), this may limit growth.

The iron requirements of infants on an exclusive milk diet are well known. If in the human, as in the rat, copper is a stimulus for hemoglobin formation, some suitable substance furnishing both iron and copper should be administered. Hoobler (30) has fed yeast to infants with good results. This may have been beneficial not only because of the additional vitamin B (complex) furnished, but because of the increased intake of copper and iron.

EXPERIMENT IV. THE SUPPLEMENTARY VALUE OF CANE AND BEET MOLASSES

It was found by Aston (3) that the use of iron ammonium citrate and molasses helped prevent or cure a disease in cattle of New Zealand. The beneficial effect of the molasses was attributed to its high iron content, analyses showing that about 7 per cent of the ash was iron oxide. Knowledge of this work led to the use of cane molasses as a supplement to an exclusive whole milk diet. A remarkably favorable response was obtained by the use of a sample of cane molasses obtained from a stock supply from which the cows in the Experiment Station herd were fed. This immediately raised the question as to whether beet molasses would respond in the same way and led to a feeding trial in which the antianemic potency of beet molasses was compared with that of cane molasses.

Weanling albino rats were allowed whole milk *ad libitum*. One group of these rats was fed 0.5 of a gram of cane molasses in a separate dish each day; and to a comparable group 0.5 of a gram of beet molasses was fed in a similar manner. To another group of rats that had been made anemic on an exclusive milk diet, 0.5 of a gram of cane molasses was fed per rat daily; and to a comparable anemic group 0.5 of a gram of beet molasses was fed daily. Hemoglobin determinations were made on each rat biweekly, and body weights were obtained weekly.

The results of this trial are shown in Figure 16. It is very obvious from the curves that the combination of milk and cane molasses allowed excellent growth and maintained the hemoglobin content of the blood at a high level, and that when cane molasses was added after severe anemia had developed, hemoglobin regeneration was restored and the blood soon became normal. On the other hand, the combination of milk and beet molasses resulted in retarded growth and did not prevent the development of anemia. Also, the addition of beet molasses to the diet of rats suffering from nutritional anemia had no beneficial effect.

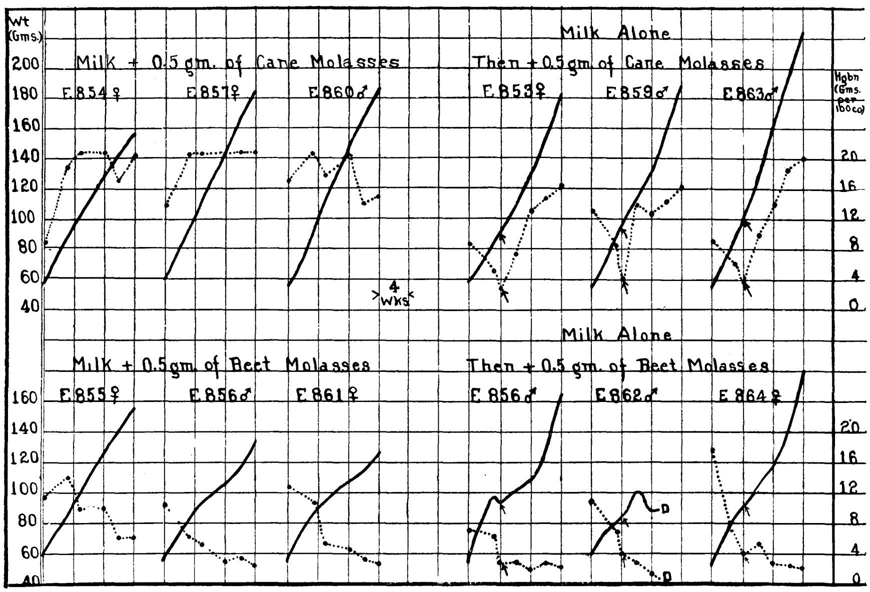

Fig. 16.—Cane molasses has considerable antianemic potency; beet molasses is practically inert in this respect. The cane molasses used contained much more iron and copper than did the beet molasses

To explain these results chemical analyses of the ash of the two kinds of molasses were made. It was found that the cane molasses contained much more iron and copper than did the beet molasses (Table 8). Since iron and copper had previously been shown to be essential for hemoglobin regeneration, it was concluded that the superiority of the cane molasses was due to its higher copper and iron content.

TABLE 8.—Chemical Composition of Cane and Beet Molasses

	Dry matter	Ash	Iron	Copper
	Pct.	*Pct.*	*Pct.*	*Pct.*
Cane molasses	70.56	5.30	0.0515	0.0286
Beet molasses	77.20	8.64	0.0114	0.0038

It must be remembered that in livestock feeding the nature of the ration is usually such as to furnish sufficient amounts of iron and copper and that thus far young pigs are the only farm animals that have been shown to suffer from nutritional anemia (26). Also, samples of cane and beet molasses obtained from different sources might differ sufficiently in chemical composition from the samples used in this trial to bring about different results.

EXPERIMENT V. THE SUPPLEMENTARY VALUE OF MANGANESE

Several laboratories have taken up the problem of nutritional anemia induced by an exclusive milk diet and have published conflicting results. On one point there is universal agreement; namely, that the addition of iron and copper will cure nutritional anemia in rats on an exclusive whole milk diet or prevent the disease if added to the milk diet from the beginning. While Waddell, Steenbock, and Hart (63) have shown copper to be a specific in the prevention of nutritional anemia produced on a diet of whole milk and iron, a vast array of other elements has been presented by other workers as having a beneficial effect on hemoglobin regeneration.

Titus, Cave, and Hughes (57) found manganese very effective and, as a result of their work, concluded: "Manganese added to a milk-iron diet seems to give almost, if not quite, as good results in the building of hemoglobin as does copper added in the same way." Myers and Beard (49) also found manganese, as well as a number of other elements, to be effective in bringing about hemoglobin regeneration in anemic rats.

The results obtained by Titus, Cave, and Hughes (57) and by Myers and Beard (49), together with the contrary results obtained by Waddell, Steenbock, and Hart (63) when they employed the same manganese salt used by the Kansas workers, made it desirable that further work be done on the role of manganese in hemoglobin regeneration. At the suggestion of Dr. E. B. Hart of the University of Wisconsin and with the approval of Dr. J. S. Hughes of the Kansas Agricultural College, it was decided to include this phase of the problem in the program on nutritional anemia being carried out in the nutrition laboratory of the Dairy Department at the Ohio Experiment Station.

EXPERIMENTAL

A sample of copper sulfate and one of manganese chloride, together with a solution of ferric chloride, were obtained from Dr. Hart. Solutions of manganous chloride and iron chloride were received from Dr. Hughes. These samples were part of the supply used previously in the respective laboratories of these workers.

The copper sulfate and manganese chloride salts from the Wisconsin laboratory were made up to contain 0.05 mg. of copper and 0.1 mg. of manganese per cc., respectively. The Wisconsin iron solution, as received, contained 5 mg. of iron per cc. This was diluted for feeding so as to contain 0.5 mg. of iron per cc. As received, the Kansas solution of iron chloride contained 15 mg. of iron per cc., and the manganous chloride solution, 5 mg. of manganese per cc. These were diluted so as to contain 0.5 mg. of iron and 0.1 mg. of manganese per cc., respectively. As no copper salt or solution was submitted by Dr. Hughes, one containing 0.05 mg. of copper per cc. was prepared from a supply of copper sulfate previously used in our own laboratory. All dilutions were made with water redistilled from glass. Qualitative tests revealed no manganese in the copper solutions and no copper in the manganese solutions.

Albino rats were used in this work. The technique of feeding, handling, and weighing the rats and of making hemoglobin determinations was the same as that previously described. For almost the entire period covered, the milk used was taken from the combined production of three Holstein cows kept and fed under normal winter feeding conditions. For a short time toward the close of the trials milk from Holstein cows having access to pasture was used.

Since the chief point under consideration in this problem was the effectiveness of a combination of iron and manganese in nutritional anemia of the rat, animals received this combination after having developed severe anemia, seven receiving additions of the Kansas salts and five of the Wisconsin salts. As it was desirable to know the effectiveness of each element alone and of various combinations other than manganese and iron, other groups of rats were so fed as to determine these points. In all, the following groupings were made, fewer animals being used in those groups on which data had been previously obtained:

Group	I—Milk exclusively										
	II—Milk	Plus	0.5	mg.	Fe	(K)					
	III—Milk	Plus	0.5	mg.	Fe	(W)					
	IV—Milk	Plus	0.05	mg.	Cu	(O)					
	V—Milk	Plus	0.05	mg.	Cu	(W)					
	VI—Milk	Plus	0.10	mg.	Mn	(K)					
	VII—Milk	Plus	0.10	mg.	Mn	(W)					
	VIII—Milk	Plus	0.5	mg.	Fe	(K)	and 0.1	mg.	Mn	(K)	
	IX—Milk	Plus	0.5	mg.	Fe	(W)	and 0.1	mg.	Mn	(W)	
	X—Milk	Plus	0.1	mg.	Mn	(K)	and 0.05	mg.	Cu	(O)	
	XI—Milk	Plus	0.1	mg.	Mn	(W)	and 0.05	mg.	Cu	(W)	
	XII—Milk	Plus	0.5	mg.	Fe	(K)	and 0.05	mg.	Cu	(O)	
	XIII—Milk	Plus	0.5	mg.	Fe	(W)	and 0.05	mg.	Cu	(W)	

(K) from laboratory of Dr. J. S. Hughes, Manhattan, Kansas
(W) from laboratory of Dr. E. B. Hart, Madison, Wisconsin
(O) from laboratory of Dr. W. E. Krauss, Wooster, Ohio

In addition, three rats were fed iron and manganese from the beginning, furnishing limited prophylactic data which, however, were entirely substantiated by previous work.

The additions were made by pipetting 1.0 cc. of each of the designated solutions into a small amount of milk for each rat at the afternoon feeding. This insured quantitative consumption. The levels of feeding the iron and copper were those previously found effective by Hart, Steenbock, Waddell, and Elvehjem (29); the level of manganese feeding was that used by Titus, Cave, and Hughes (57).

RESULTS

The results are shown graphically in the charts. Curves for only four control animals are shown (Fig. 17), but during the course of this work many more rats developed nutritional anemia on the same milk. The addition of iron from either source, in four cases out of six, slightly raised the hemoglobin temporarily and prolonged life for several weeks (Fig. 17). Eventually, however, the rats died. When copper was added, the hemoglobin was raised considerably, growth was continued, and life was indefinitely prolonged (Fig. 18). These effects of the addition of iron and copper, respectively, have been previously observed in this laboratory.

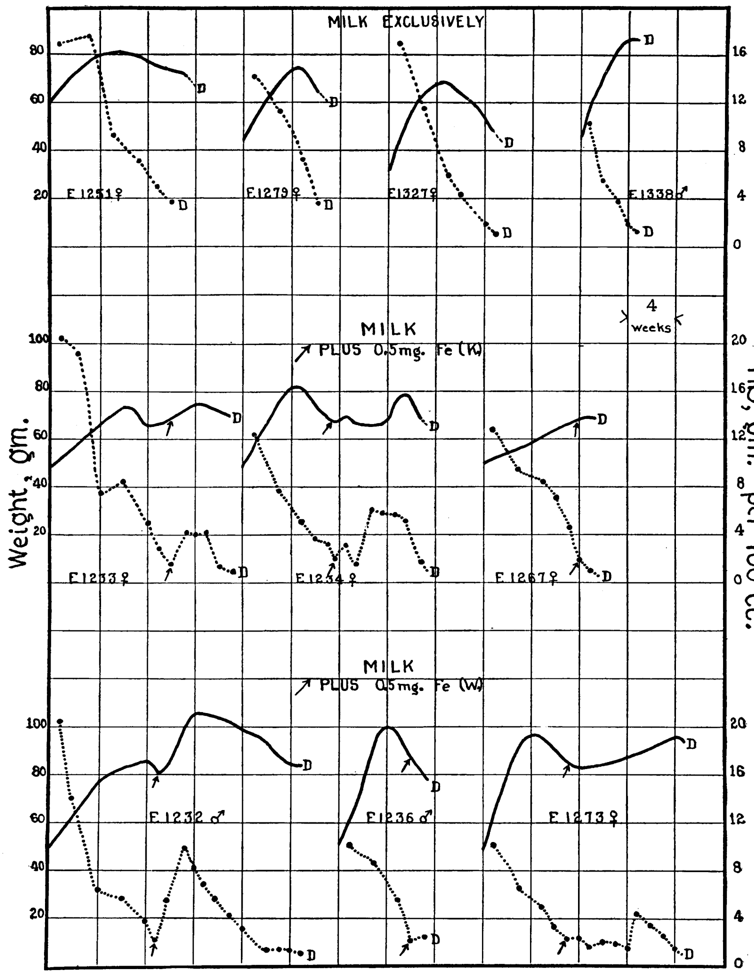

Fig. 17.—The effects of an exclusive milk diet and of the addition of Fe (K) and Fe (W) are shown by these curves. Both iron salts brought about a temporary stimulation in hemoglobin production.

The addition of manganese from either source did not stimulate hemoglobin regeneration, nor did it prevent the decline in hemoglobin and ensuing death (Fig. 18). When iron and manganese were added simultaneously the response was similar to that obtained by the addition of iron alone and was probably due to the iron only (Figs. 19 and 20). Attention is called to Rat E 1231 ♂, which received, after its hemoglobin had fallen to 2.0 grams per

100 cc. of blood, the regular additions of iron and manganese. After a further drop in hemoglobin during the following week, sudden improvement in the animal was observed. This continued for three weeks until it was discovered that attached to the cage was a piece of copper wire which had obviously been gnawed by the rat. This wire was removed, and in the course of two weeks the rat was developing anemia. That the rat developed anemia in the first place was probably due to the fact that it had not discovered the wire.

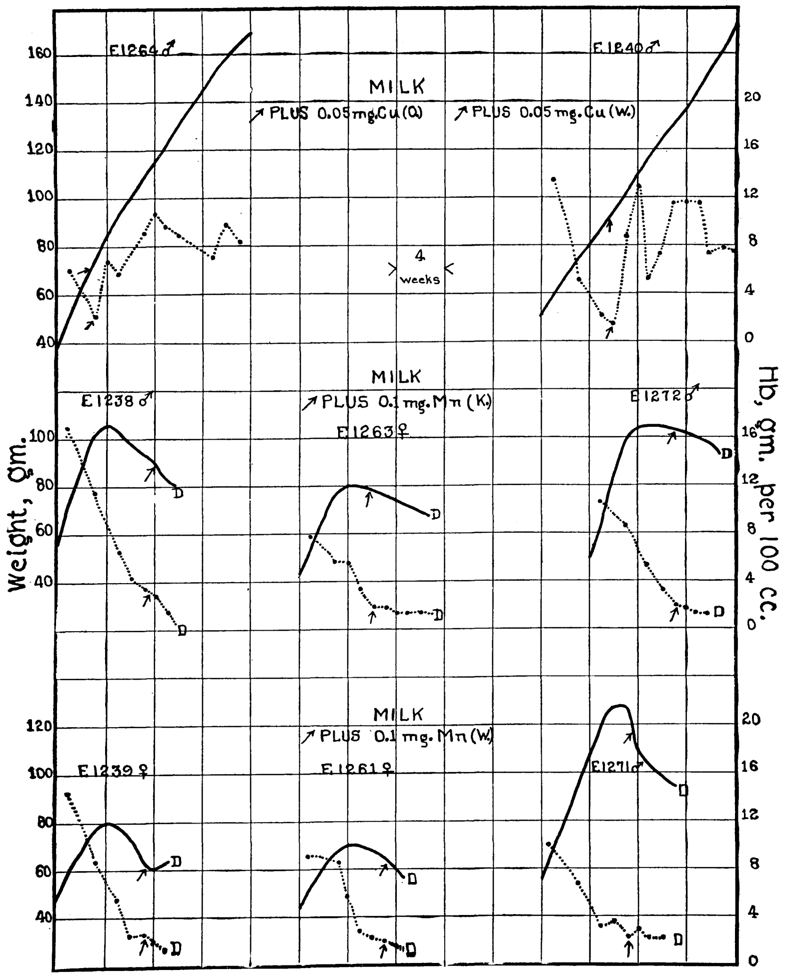

Fig. 18.—Some response was obtained by the addition of copper alone. The addition of manganese from either source did not prevent a continued drop in hemoglobin

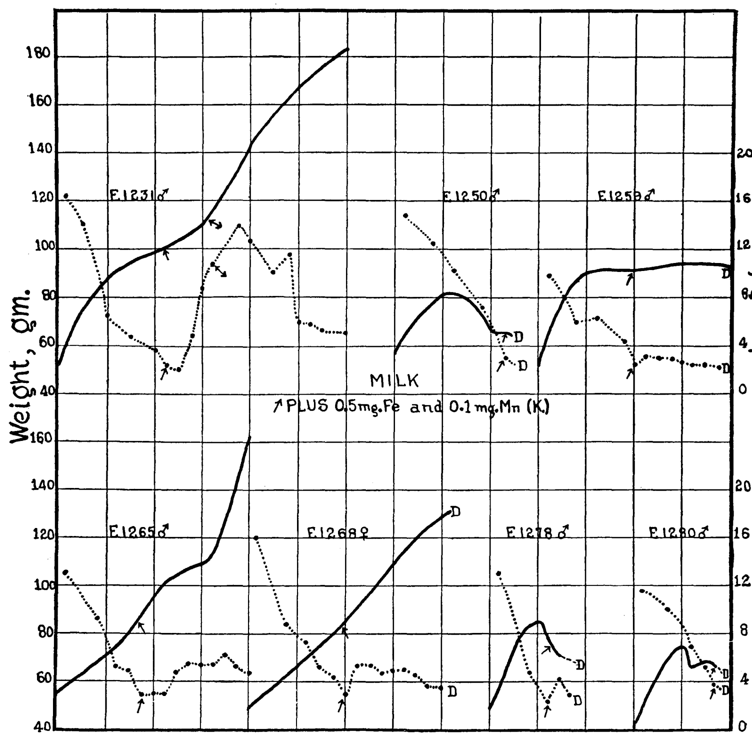

Fig. 19.—The ineffectiveness of Fe (K) and Mn (K) combined is shown here. The slight response obtained upon the addition of Fe and Mn was no greater than that obtained by the addition of Fe alone (Fig. 17) and was probably due only to the iron.

Supplements to deficient diets are usually more effective as prophylactics than as curatives. However, when iron and manganese were added simultaneously from the time the rats were placed on milk, anemia developed with the same rapidity as when the usual milk-iron diet was fed. In addition to the data represented by the curves for Rats E 1405, E 1406, and E 1407, it had been found in this laboratory previously that the addition of manganese sulfate to a milk diet did not prevent anemia, nor did the addition of the Daniels and Hutton salt mixture[3], which contains manganese (11).

[3] $AlK(SO_4)_2$. 12 H_2O; NaF; Na_2SiO_3; $MnSO_4$. 4 H_2O. Fed so that each animal received 1.5 mg. of each salt daily. 1 drop of a 2 per cent solution of sodium iodide and 3 drops of a saturated solution of iron citrate were added to each 200 cc. of milk.

The combination of copper and manganese produced the same result as when copper alone was added (Fig. 21). On the other hand, when copper and iron were fed simultaneously, the amount of copper being only half the amount of manganese fed, the response was immediate and phenomenal. The remarkable effect of copper is emphasized by the reaction of Rat E 1241 (Fig. 21). When the hemoglobin level of this animal had dropped to 4.0 grams per 100 cc. of blood, the animal was not only suffering from severe anemia but from an acute intestinal infection. This animal could well have been discarded as its death was imminent, but copper and iron were administered and in the course of three weeks the rat was apparently a normal individual.

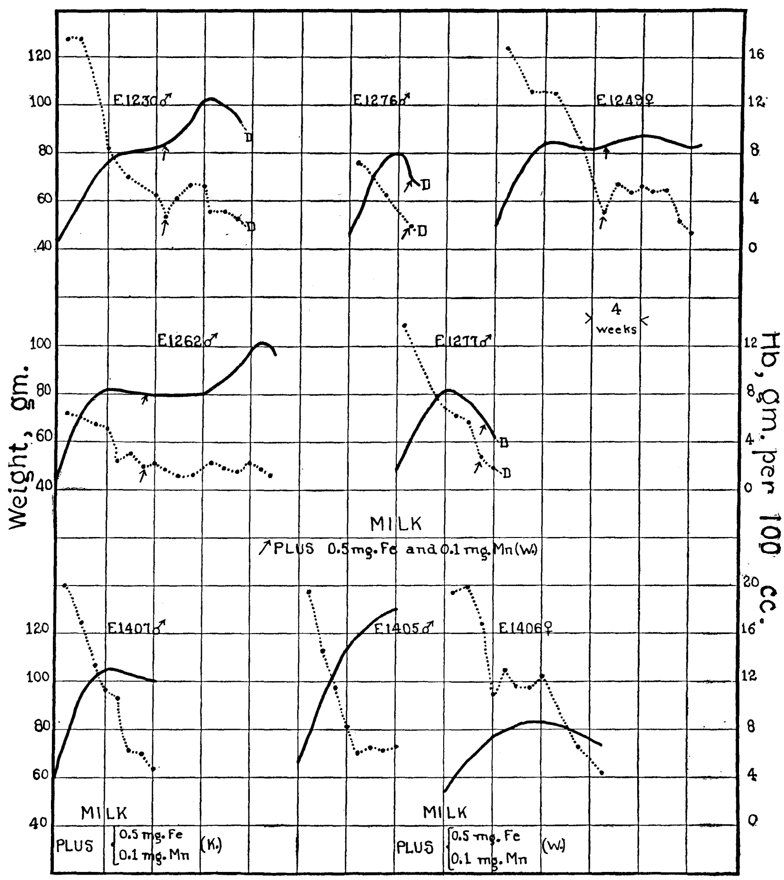

Fig. 20.—The ineffectiveness of Fe (W) and Mn (W) combined, as a curative, and of salts from both sources as prophylactics, is emphasized here

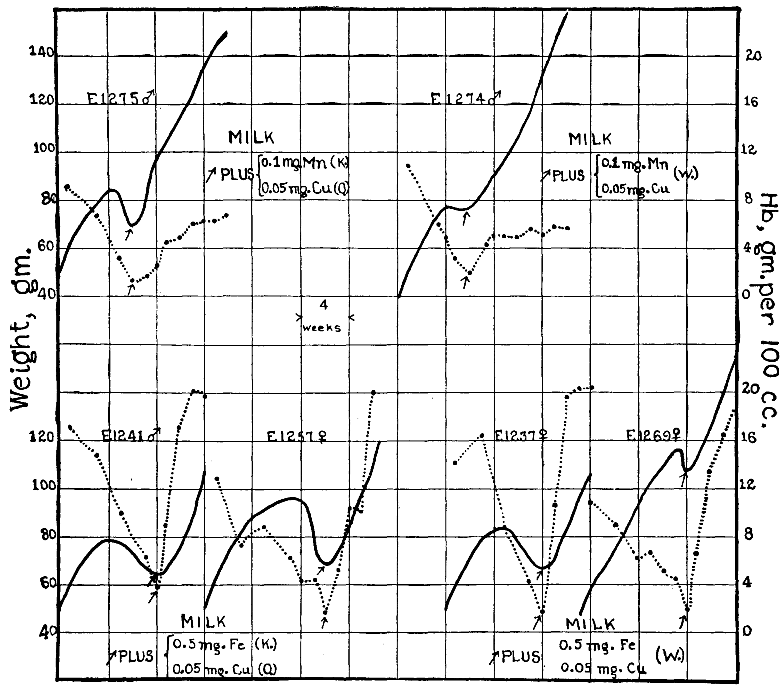

Fig. 21.—The combination of Mn and Cu was no more effective than was Cu alone, as shown here. Fe and Cu, fed simultaneously, resulted in phenomenal response.

DISCUSSION

A study of the graphs reveals that no difference in response resulted when salts prepared in different laboratories were fed simultaneously to rats of the same litter. The fact that neither the Wisconsin workers nor we could demonstrate any beneficial effect through the use of iron and manganese salts from the same source as those used by Titus, Cave, and Hughes would indicate that some contamination existed in the materials in the Kansas laboratory. This theory is strengthened by the inability of Hughes[4] to repeat the results first obtained.

The ease with which a contamination may creep into this type of work is demonstrated by our experience with Rat E 1231, reported in this experiment (Fig. 19), and with a few others. The source of contamination may not always be detectable. In one instance we found it impossible to make a group of four rats,

[4]Personal communication from Dr. Hughes.

housed in one cage, anemic on an exclusive milk diet. A close examination of the cage and all equipment revealed nothing; however, when the rats were removed to another cage they promptly developed anemia. The desirability of using glass equipment throughout is apparent as this would preclude the possibility of metallic contamination.

The argument may be advanced that the milk used in our work contained less manganese than that used by Titus, Cave, and Hughes. Analysis of a preserved composite sample of the milk used in this trial was made in the laboratory of Dr. Hart. It was found to contain 0.034 mg. of manganese per liter[5], as compared to 0.020 mg. per liter in milk sent to the Wisconsin laboratory by Dr. Hughes. It is not likely that a deficiency of manganese in the milk used by us could be responsible for the lack of response when 0.1 mg. of manganese was added daily. Further refutation of this argument is advanced by the work of Waddell, Steenbock, and Hart (63), who fed as high as 5.0 mg. of manganese per rat per day without effect.

The phenomenal responses we have obtained by the addition of copper under extremely adverse conditions has emphasized the role played by this element as a specific for hemoglobin regeneration in rats suffering from nutritional anemia.

Further evidence of the specificity of copper and the inadequacy of manganese was offered by Lewis, Weichselbaum, and McGhee (35) who found electrolytically prepared iron and copper effective while manganese prepared from manganese sulfate (C. P. Baker's) and fed with the electrolytically prepared iron, produced no response.

Myers and Beard (49) and Beard and Myers (5) reported favorable responses through the use of iron, copper, nickel, germanium, arsenic, manganese, cobalt, titanium, zinc, rubidium, vanadium, chromium, selenium, or mercury.

Recent work of Mitchell and Miller (46) also indicates that a number of inorganic elements may be concerned. While no criticism of the technique of these workers is intended or can be justly made without further investigation, it is difficult to conceive how so many substances can be concerned in the performance of so specific a function as hemoglobin regeneration.[6]

[5]Analysis of the fresh milk used in our work gave somewhat higher results, ranging from 0.042 to 0.059 mg. per liter.

[6]Since the preparation of this manuscript, Underhill, Orten, and Lewis have published a paper (Jour. Biol. Chem. 91: 13-25. 1931) showing that cobalt, nickel, zinc, and manganese as supplements to iron fail to cure nutritional anemia in rats. Purified iron alone was also ineffective. In this work glass equipment was used throughout.

EXPERIMENT VI. REPRODUCTION ON MILK DIETS

In the preliminary work with exclusive milk diets it soon became evident that no data on reproduction could be obtained. Most of the rats died before reaching sexual maturity, and the males that survived long enough to become sexually mature developed priapism. When the beneficial supplementary value of iron and copper became known, the question arose as to whether or not additions of iron and copper to a whole milk diet would allow completion of the normal life cycle.

Mattill and Conklin (37) observed that when weanling rats were placed upon a liquid milk diet no reproduction occurred even though good growth was obtained for 50 to 100 days; neither did the use of dried milk or of a ration consisting mostly of dried skimmilk result in reproduction. Examination of the gonads showed that these organs were, with one exception, normal in weight; however, the ovaries were 50 per cent or more below normal in weight. These investigators stated at that time that possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth and reproduction, especially in the female.

Later, Mattill and Stone (38) failed to obtain reproduction in rats receiving rations in which all the protein and known vitamins were supplied by varying proportions of dry milk. A change to rations containing added protein-free milk, cod-liver oil, traces of potassium iodide, or even to stock rat food did not restore the reproductive function. These results were attributed to unthriftiness of the strain of rats used.

In subsequent work Mattill, Carman, and Clayton (36) showed that a milk diet high in fat did not allow reproduction in rats, but that when a milk diet low in fat was used marked reproductive failure did not appear. The trouble was entirely eliminated by the use of 5 to 10 per cent of wheat embryo which led to the conclusion that the factor X, now known as vitamin E, was lacking.

Anderegg and Nelson (2) obtained reproduction in rats fed diets made up largely of whole milk powder and supplemented with iron salts. A corresponding skimmilk powder diet did not allow reproduction unless wheat embryo or yeast was added. This indicated that a factor found in the fat of milk was concerned in reproduction.

Daniels and Hutton (11) overcame the reproductive difficulty accompanying exclusive milk diet feeding by using a complex mineral supplement containing aluminum, potassium, sodium, fluorine, silicon, manganese, iron, and iodine.

Keil and Nelson (33) found that rats on a diet of whole milk, wheat embryo, and ferric chloride did not reproduce. However, when copper sulfate was added normal reproduction resulted. Lactation was fair.

Waddell, Steenbock, and Hart (64) also found that rats reproduced quite well on a diet of whole milk, iron, and copper, but that lactation was poor. The addition of manganese and iron improved lactation considerably.

EXPERIMENTAL

Weanling albino rats were so distributed that two males and two females were in each cage and allowed every opportunity to breed. A small enough quantity of milk containing sufficient copper sulfate and colloidal ferric oxide to allow 0.5 mg. of iron and 0.16 mg. of copper[7] for each rat, assuming equal milk consumption per individual, to permit complete consumption overnight was placed in a glass beaker in each cage in the late afternoon. In the morning the rats were given free access to milk.

Males and females were left together for at least a year. When pregnancy could be detected the pregnant animals were moved to individual cages for parturition and were left with the young until weaning time, which occurred on the twenty-fourth day. During this time the mother was fed the usual milk diet supplemented with copper and iron. The young were weighed at birth and weekly thereafter. At weaning time two males and two females were selected and placed in a cage from which time on the procedure was the same as that carried out with the first generation.

As it had been previously shown that yeast and cane molasses possessed considerable antianemic potency these substances were used as supplements in the reproduction study. Wheat germ oil was also used since it was known to be potent in vitamin E.

RESULTS

For several months after the first group of rats was started on this experiment no reproduction occurred. This was attributed to seasonal influence as the rats became sexually mature in the fall at

[7]Later in the experiment these levels were reduced to 0.25 mg. of iron and 0.08 mg. of copper.

which time of the year, as measured by the number of litters born, there has been observed in our stock colony a marked decline in reproductive activity. This theory was upheld by establishing, after unitesticular castration, the presence of sperm in the epididymis and the motility of the sperm after stimulation with 0.85 per cent sodium chloride.

In the spring of the first year of the experiment the first litters were born; and from then on litters were born at frequent intervals, the fewest being born in the fall.

The pertinent results are summarized in Table 9. The table shows that reproduction occurred, but that the number of young reared was small. A few fourth generation rats have been obtained on milk plus iron and copper, but these individuals are quite subnormal in size and general thriftiness.

TABLE 9.—Reproduction on an Exclusive Whole Milk Diet and the Same Diet Variously Supplemented

Diet	Rats		No. of litters	No. of live young	Young raised	Per cent raised
	Male	Female				
Milk alone	28	32	....	....	....	....
Milk plus 0.16 mg. Cu	3	6	3	19	8	42.1
Milk plus 0.16 mg. Cu and 0.5 mg. Fe	31	38	25	160	38	23.8
Milk plus Cu and Fe and 0.5 gram wheat germ oil	8	8	16	114	38	33.3
Milk plus yeast	3	3	2	14	....	....
Milk plus yeast and Cu and Fe	4	4	12*	75	36	48.0
Milk plus 0.5 gm. cane molasses	3	6	4	23	7	30.4

*Includes two cases where litters were apparently born but no young were found.

Reproduction occurred when yeast or cane molasses was added to the milk diet, but, as in the case of the other additions, lactation was very poor.

On the whole, reproduction was in no case normal. The young were born without difficulty but were slightly lighter in weight than those from stock females. No greater mortality occurred at birth than would normally be expected. Young were born at irregular intervals in spite of the fact that the females were replaced with the males on the same day the litters were weaned.

DISCUSSION

The fact that reproduction occurred on a whole milk diet properly fortified with iron and copper salts or with substances shown to have considerable antianemic potency indicates that milk contains vitamin E and that the maintenance of hemoglobin at a high level simply enables the rats to attain sexual maturity. Some of the results of other workers who obtained second and third generation animals on a diet of milk and rice or milk and bread may have been due to the fact that the rats were not placed on these diets until after they had a sufficient storage of iron and copper to carry them over the critical adolescent period.

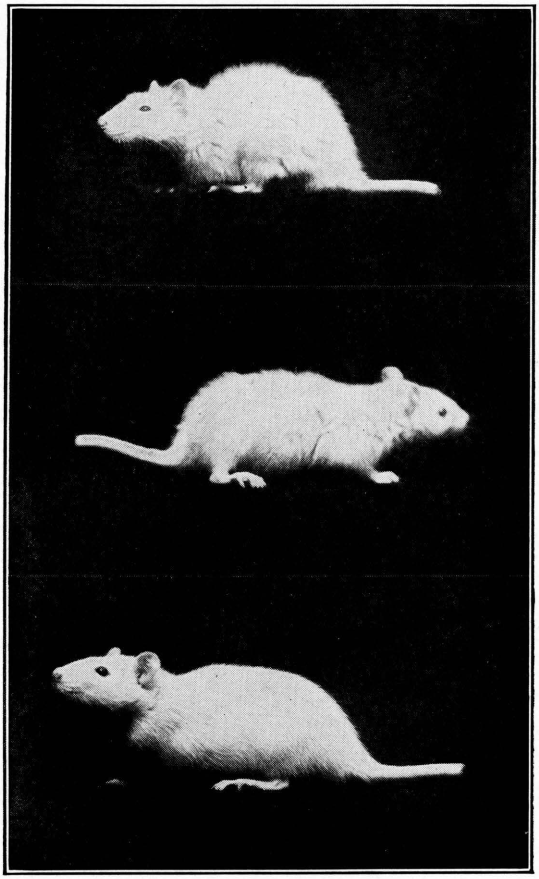

Fig. 22.—These three rats were brought to the same stage of anemia by feeding an exclusive whole milk diet. The rat at the top then received 0.5 mg. of iron daily and the one in the center 0.5 mg. of iron and 0.1 mg. of manganese, without improvement. The rat at the bottom received 0.5 mg. of iron and 0.05 mg. of copper and soon became normal with respect to hemoglobin.

It is apparent that milk, in addition to being deficient in iron and copper, is lacking in a factor or factors necessary for lactation. That manganese and iodine may be involved is indicated by the work of Waddell, Steenbock, and Hart (64). It must be remembered too that while Daniels and Hutton (11) stressed the importance of aluminum, silicon, manganese, and fluorine, they added as a matter of routine iodine and iron, and that some copper contamination of the salts used might be assumed.

GENERAL DISCUSSION

It is evident that the term "perfect food" can no longer be applied to milk. A perfect food would be one that could support normal life indefinitely. As shown in the experiments reported here, milk is deficient in iron and copper, both of which are essential for maintaining the hemoglobin content of the blood at a normal level.

The question immediately arises as to whether or not the iron and copper content of milk can be increased by feeding greater quantities of these minerals than are ordinarily found in the cow's ration. It has been demonstrated by Elvehjem, Herrin, and Hart (19), Elvehjem, Steenbock, and Hart (21), and by Hamilton, Mitchell, and Nevens (25) that the iron and copper content of milk cannot be increased by feeding iron and copper salts to the cow.

The deficiency of milk concerned with lactation, as demonstrated by the reproduction experiments, is of no serious consequence inasmuch as the lactating animal is ordinarily on a diet of such a nature as to allow satisfactory lactation.

PRACTICAL CONSIDERATIONS

HUMAN FEEDING

While results obtained with small animals cannot always be interpreted in terms of humans, the fundamental facts remain. However, it has been shown that iron and copper are effective in certain secondary anemias in infants in much the same way as they are in nutritional anemia of the rat (45). At the General Hospital in Montreal, Canada, 10 cases of anemia of long standing which had not yielded to iron treatment were immediately improved by the addition of copper sulfate (45).

Based upon analyses of infant livers, the child is born with a supply of iron and copper sufficient to meet its needs for some time. However, it is now recognized as good practice to include some food like strained spinach in the infant's diet as soon as possible.

Using the accepted technique for studies in nutritional anemia, Daniels and Forman (10) found that carbohydrate milk-modifiers differ in their ability to bring about hemoglobin formation. Dextri-Maltose, corn syrup, vitavose, or Mellin's food, when fed at a 7 per cent level with milk, gave hemoglobin values slightly below optimum. Orange juice and tomato juice had no significant effect on hemoglobin. With infants, wheat embryo extract proved to be very effective.

As soon as the human diet becomes varied no further concern as to copper and iron deficiencies need be felt, provided a certain amount of care is exercised in the selection of foods. Some internal organ, such as liver or kidney, should be used occasionally as a source of meat. As a guide in the selection of foods the following list has been compiled, based upon hemoglobin regeneration studies with dogs made at the University of Rochester Medical School.[8]

GOOD	MODERATE	POOR
Beef liver	Beef muscle	Green vegetables
Calf liver	Beef heart	Fish liver
Chicken liver	Pig muscle	Milk
Pig liver	Pig heart	Cream
Chicken gizzard	Dried apricots	American cheese
Pig kidney	Dried peaches	Butter
Beef kidney	Dried prunes	Fresh black raspberries
	Bone marrow	Onions
	Spleen	Orange juice
	Brains	Egg yolk
	Pancreas	Egg white
	Raisins	
	Fresh grapes	
	Fresh apples	
	Dried apples	
	Spinach	
	Cabbage	
	Liver sausage	
	Blood sausage	
	Veal	
	Chicken	
	Gelatin	

Of particular significance in this table is the position which dairy products and green vegetables hold with respect to hemoglobin-forming power. It is ordinarily considered that green vegetables and dairy products, particularly milk, are the principal protective foods. This applies particularly to their mineral and vitamin content, and the fact that in anemia they are of very little

[8]This list has been approved by Dr. G. H. Whipple of the University of Rochester Medical School, to whom grateful acknowledgment is here extended.

value detracts in no way from their importance. In fact, the well-balanced diet must include them. In this connection Whipple and Robscheit-Robbins (65) say: "Whole milk stands at the foot of the class of diet factors which bring about hemoglobin regeneration in severe anemia. Excellent as milk is for most dietary requirements, it is conspicuously inadequate in hemoglobin-producing ingredients. Physicians should keep this in mind in diet control of anemia, especially in young children or infants where milk is to be the main diet constituent."

DAIRY PRODUCTS

The low antianemic potency of milk, cream, American cheese, and butter has already been pointed out. Desiccated milk, however, has been shown to have considerable antianemic potency, the factor responsible for this being imparted in the drying process through contact with metals. Supplee, Dow, Flanigan, and Kahlenberg (52) have shown that milk dried between heated metal rollers contains considerably more iron than does the original liquid milk; while Titus and Hughes (58) have shown that the copper content of milk is increased in the drying process. Dried and condensed milk have been shown by Randoin and Lecoq (50) to prevent anemia in rats when constituting the entire diet.

ANIMAL FEEDING

In the field of animal feeding it is known that various types of anemia occur in horses, cattle, sheep, and swine. So-called chlorosis in sheep, which develops when the animals are pastured in marshy moorland pastures, or after failures of crops, or if the animals are fed in winter insufficiently on poor foodstuffs such as beet leaves, may be assumed to be anemia due to faulty nutrition. Anemia or hydremia occurs with relative frequency in cattle kept near alcohol distilleries or sugar refineries. In New Zealand "bush-sickness" in cattle and sheep, characterized by pronounced anemia, has been traced to an iron deficiency (3, 24).

That a large number of suckling pigs kept indoors develop severe anemia has been shown by McGowan and Crichton (41, 42), and by Doyle, Mathews, and Whiting (13). Not until after the discovery of the role of iron and copper in nutritional anemia of the rat, however, was the cause or remedy for this ailment found. It was found by Hart, et al. (26) that anemia could be prevented in young pigs by giving them access to iron and copper. It was later found that iron alone was sufficient. Some readily soluble iron salt can be fed to the pigs directly or an iron solution may be painted on the sow's teats each day. The pigs are not benefited by feeding the sow additional iron.

So far as is now known no other class of farm animal in this country requires additional quantities of iron and copper to those found in ordinary rations.

GENERAL SUMMARY

Rats fed on liquid whole milk exclusively, the milk coming from Holstein cows under winter feeding conditions and receiving a good dairy ration, developed nutritional anemia and died. The milk used contained 2.4 mg. of iron and 0.5 mg. of copper per liter.

The addition of ferrous sulfate, ferric citrate, ferric chloride, or colloidal ferric oxide to an exclusive milk diet was ineffective in preventing nutritional anemia.

The addition of a small amount of copper (0.16 mg. daily) as copper sulfate was quite beneficial.

The addition of 0.2 mg. of iron and 0.16 mg. of copper, or of 0.4 mg. of iron and 0.32 mg. of copper proved highly effective in preventing nutritional anemia in rats.

Yeast, casein, agar, and McCollum's salt mixture 185 were found effective, the degree of effectiveness depending in general upon the amount of copper and iron furnished by each substance.

Cod-liver oil, gelatin, rice polishings, wheat germ oil, and irradiated milk were ineffective.

Seven samples of yeast showed definite hemoglobin regencrating power of varying degrees; three samples showed very little or none. Some correlation appeared to exist between the copper content of the yeasts and their antianemic potency. No such correlation was evident with respect to iron.

Cane molasses was found to possess great antianemic potency; while beet molasses was practically inert. The superiority of cane molasses was attributed to its higher iron and copper content.

Manganese, when added alone (0.1 mg.) or in combination with iron (0.5 mg.), did not bring about improvement in rats suffering from nutritional anemia.

No reproduction occurred in rats fed an exclusive whole milk diet. The addition of copper; iron and copper; iron, copper, and yeast; iron, copper, and wheat germ oil; yeast alone; or cane molasses allowed reproduction, but lactation was poor.

REFERENCES CITED

1. Abderhalden, E. Die resorption des Eisens, sein verhalten im Organismus und seine ausscheidung. Zeitschr. Biol. **39**: 113-252. 1900.
2. Anderegg, L. T. and V. E. Nelson. Whole and skimmed milk powders as food. Observations on a new vitamin for reproduction. Jour. Ind. Eng. Chem. **17**: 451-455. 1925.
3. Aston, B. C. Iron-hunger in ruminant stock. N. Z. Jour. Agr. **30**: 175-186. 1925.
4. Beard, H. H. and V. C. Myers. Blood regeneration in nutritional anemia. Influence of iron, iron and copper, nickel, cobalt, germanium or sodium germanate. Proc. Soc. Exp. Biol. and Med. **26**: 510-11. 1929.
5. Beard, H. H. and V. C. Myers. Further observations on the effect of inorganic elements in nutritional anemia. Jour. Biol. Chem. **87**: p. xxxix-xl. 1930.
6. Biazzo, R. Metodo rapido di determinazione del rame nelle conserve di prodotti vegetali rinverditi. Annali di Chimica Applicata. **16**: Fasc. 2, Febbraio, 1926.
7. Brouwer, E. Note on anaemia, urobilinuria and intestinal haemorrhage in rabbits in consequence of exclusive nutrition with cow's and goat's milk. Biochem. Jour. **20**: 105-107. 1926.
8. Bunge, G. Ueber die Aufnahme des Eisens in den Organismus des Säuglings. Zeitschr. Physiol. Chem. **13**: 399-406. 1889.
9. Cartland, G. F. and F. C. Koch. Diet proteins and vitamins as related to hemoglobin production in the rat. Amer. Jour. Physiol. **87**: 249-261. 1928.
10. Daniels, A. L. and M. B. Forman. Relation of various substances used in the artificial feeding mixtures of infants to nutritional anemia. Proc. Soc. Exp. Biol. and Med. **28**: 431-432. 1931.
11. Daniels, A. L. and M. K. Hutton. Mineral deficiencies of milk as shown by growth and fertility of white rats. Jour. Biol. Chem. **63**: 143-156. 1925.
12. Donaldson, H. H. The Rat. 2nd Edition, Tables 145 and 146. 1924.
13. Doyle, L. P., F. P. Mathews, and R. A. Whiting. Anemia in young pigs. Purdue Univ. Agr. Exp. Sta. Bull. 313. 1927.
14. Drabkin, D. L. and H. K. Miller. Hemoglobin production. II. The relief of anemia, due to milk diet, by feeding amino acids. Jour. Biol. Chem. **90**: 531-543. 1931.
15. Drabkin, D. L. and C. S. Waggoner. Hemoglobin maintenance upon synthetic diets. Science **69**: 480. 1929.
16. Drabkin, D. L. and C. S. Waggoner. Hemoglobin maintenance and production upon synthetic diets, including modifications in the ethyl xanthate and Biazzo methods for copper analysis. I. Jour. Biol. Chem. **89**: 51-75. 1930.
17. Elvehjem, C. A. and E. B. Hart. Iron in nutrition. II. Quantitative methods for the determination of iron in biological materials. Jour. Biol. Chem. **67**: 43-51. 1926.
18. Elvehjem, C. A. and E. B. Hart. The relation of iron and copper to hemoglobin synthesis in the chick. Jour. Biol. Chem. **84**: 131-141. 1929.
19. Elvehjem, C. A., R. C. Herrin, and E. B. Hart. Iron in nutrition. III. The effects of diet on the iron content of milk. Jour. Biol. Chem. **71**: 255-262. 1927.
20. Elvehjem, C. A. and C. W. Lindow. The determination of copper in biological materials. Jour. Biol. Chem. **81**: 435-443. 1929.
21. Elvehjem, C. A., H. Steenbock, and E. B. Hart. The effect of diet on the copper content of milk. Jour. Biol. Chem. **83**: 27-34. 1929.
22. Forman, M. B. and A. L. Daniels. Effect of nutritional anemia on size of the heart. Proc. Soc. Exptl. Biol. and Med. **28**: 479-480. 1931.
23. Fowweather, F. S. The determination of iron in blood, tissues and urine. Biochem. Jour. **20**: 93-98. 1926.

24. Grimmet, R. E. R. and B. W. Simpson. Soil and pasture in relation to pining and bush-sickness of sheep. Trans. Proc. New Zealand Inst. 59: 395-403. 1928.

25. Hamilton, T. S., H. H. Mitchell, and W. B. Nevens. Illinois Agr. Exp. Sta. Ann. Rept., 1928-29, p. 120. 1929.

26. Hart, E. B., C. A. Elvehjem, H. Steenbock, G. Bohstedt, and J. M. Fargo. A study of the anemia of young pigs and its prevention. Jour. Nutrition 2: 277-294. 1930.

27. Hart, E. B., C. A. Elvehjem, J. Waddell, and R. C. Herrin. Iron in nutrition IV. Nutritional anemia on whole milk diets and its correction with the ash of certain plant and animal tissues or with soluble iron salts. Jour. Biol. Chem. **72**: 299-320. 1927.

28. Hart, E. B., H. Steenbock, C. A. Elvehjem, and J. Waddell. Iron in nutrition. I. Nutritional anemia on whole milk diets and the utilization of inorganic iron in hemoglobin building. Jour. Biol. Chem. **65**: 67-80. 1925.

29. Hart, E. B., H. Steenbock, J. Waddell, and C. A. Elvehjem. Iron in nutrition. VII. Copper as a supplement to iron for hemoglobin building in the rat. Jour. Biol. Chem. **77**: 797-812. 1928.

30. Hoobler, B. R. Symptomatology of vitamin B deficiency in infants. Jour. Amer. Med. Assoc. **91**: 307-310. 1928.

31. Hunt, C. H. and W. E. Krauss. The relative antineuritic and antipellagric potency of cow's milk. Jour. Biol. Chem. **79**: 733-738. 1928.

32. Jones, J. H. The relation of the inorganic constituents of a ration to the production of ophthalmia in rats. Jour. Biol. Chem. **75**: 139-146. 1927.

33. Keil, H. L. and V. E. Nelson. Role of copper in hemoglobin formation. Proc. Soc. Exptl. Biol. and Med. **28**: 392-393. 1931.

34. Krauss, W. E. The food value of milk as affected by rations. The effect of high and low protein rations. (Progress report). Jour. Dairy Sci. **11**: 105. 1928.

35. Lewis, G. T., T. E. Weichselbaum, and J. L. McGhee. Effect of metals purified by electrolytic deposition on hemoglobin regeneration in anaemic white rat. Proc. Soc. Exptl. Biol. and Med. **27**: 329-331. 1930.

36. Mattill, H. A., J. S. Carman, and M. M. Clayton. The nutritive properties of milk. III. The effectiveness of the X substance in preventing sterility in rats on milk rations high in fat. Jour. Biol. Chem. **61**: 729-740. 1924.

37. Mattill, H. A. and R. E. Conklin. The nutritive properties of milk, with special reference to reproduction in the albino rat. Jour. Biol. Chem. **44**: 137-158. 1920.

38. Mattill, H. A. and N. C. Stone. The nutritive properties of milk with special reference to reproduction in the albino rat. II. Jour. Biol. Chem. **55**: 443-455. 1923.

39. McCollum, E. V., Nina Simmonds, and J. E. Becker. On a type of ophthalmia caused by unsatisfactory relations in the inorganic portion of the diet. An ophthalmia not due to starvation for fat-soluble A, and not curable by its administration. Jour. Biol. Chem. **53**: 313-321. 1922.

40. McCollum, E. V., Nina Simmonds, and J. E. Becker. Further studies on the cause of ophthalmia in rats produced with diets containing vitamin A. Jour. Biol. Chem. **64**: 161-179. 1925.

41. McGowan, J. P. and A. Crichton. On the effect of deficiency of iron in the diet of pigs. Biochem. Jour. **17**: 204-207. 1923.

42. McGowan, J. P. and A. Crichton. Iron deficiency in pigs. Biochem. Jour. **18**: 265-272. 1924.

43. McHargue, J. S., D. J. Healy, and E. S. Hill. The relation of copper to the hemoglobin content of rat blood. Preliminary report. Jour. Biol. Chem. **78**: 637-641. 1928.

44. McKay, C. M. The influence of protein, blood, liver, fat, iron and potassium in the diet upon the rate of blood regeneration after hemorrhage in the rat and dog. Amer. Jour. Physiol. **84**: 16-35. 1928.
45. Mendenhall, D. R. Wisconsin Agr. Exp. Sta. Bull. 420, p. 75. 1931.
46. Mitchell, H. S. and L. Miller. Inorganic elements of spinach in the treatment of nutritional anemia. Jour. Biol. Chem. **85**: 355-363. 1929.
47. Mitchell, H. S. and L. Schmidt. The relation of iron from various sources to nutritional anemia. Jour. Biol. Chem. **70**: 471-486. 1926.
48. Mitchell, H. S. and M. Vaughn. The relation of inorganic iron to nutritional anemia. Jour. Biol. Chem. **75**: 123-137. 1927.
49. Myers, V. C. and H. H. Beard. The influence of inorganic elements on blood regeneration in nutritional anemia. Jour. Amer. Med. Assoc. **93**: 1210-1212. 1929.
50. Randoin, Mrs. L. and R. Lecoq. Does exclusive consumption of milk always produce anemia in rats? Bull. Soc. Hyg. Aliment. 18, 355-356. 1930. (Abstracted in Chem. Absts. 25: 729. 1931)
51. Scott, J. M. D. Studies in anaemia. II. Biochem. Jour. **17**: 166-169. 1923.
52. Supplee, G. C., O. D. Dow, G. E. Flanigan, and O. J. Kahlenberg. A comparative study of liquid and dry milk as anemia-producing diets. Jour. Nutrition **2**: 451-469. 1930.
53. Sure, B. and M. C. Kik. Influence of the maternal diet on concentration of hemoglobin of nursing young of albino rat. Proc. Soc. Exptl. Biol. and Med. **26**: 603-605. 1929.
54. Sure, B., M. C. Kik, and M. E. Smith. Hematopoietic function in avitaminosis. VI. Vitamin G deficiency. Proc. Soc. Exptl. Biol. and Med. **28**: 498-499. 1931.
55. Sure, B., M. C. Kik, and D. J. Walker. Blood formation of the albino rat on a standard dietary régime. Jour. Nutrition **1**: 299-310. 1929.
56. Sure, B., M. C. Kik, and D. J. Walker. The effect of avitaminosis on hematopoietic function. II. Vitamin B deficiency. Jour. Biol. Chem. **83**: 387-400. 1929.
57. Titus, R. W., H. W. Cave, and J. S. Hughes. The manganese-copper-iron complex as a factor in hemoglobin building. Jour. Biol. Chem. **80**: 565-570. 1928.
58. Titus, R. W. and J. S. Hughes. The nutritional value of copper in powdered whole milk. Jour. Dairy Sci. **12**: 90-93. 1929.
59. Waddell, J., C. A. Elvehjem, H. Steenbock, and E. B. Hart. Iron in nutrition. VI. Iron salts and iron-containing ash extracts in the correction of anemia. Jour. Biol. Chem. **77**: 777-795. 1928.
60. Waddell, J., H. Steenbock, C. A. Elvehjem, and E. B. Hart. Iron in nutrition. V. The availability of the rat for studies in anemia. Jour. Biol. Chem. **77**: 769-775. 1928.
61. Waddell, J., H. Steenbock, C. A. Elvehjem, and E. B. Hart. Iron in nutrition. IX. Further proof that the anemia produced on diets of whole milk and iron is due to a deficiency of copper. Jour. Biol. Chem. **83**: 251-260. 1929.
62. Waddell, J., H. Steenbock, and E. B. Hart. Iron in nutrition. VIII. The ineffectivness of high doses of iron in curing anemia in the rat. Jour. Biol. Chem. **83**: 243-250. 1929.
63. Waddell, J., H. Steenbock, and E. B. Hart. Iron in nutrition. X. The specificity of copper as a supplement to iron in the cure of nutritional anemia. Jour. Biol. Chem. **84**: 115-130. 1929.
64. Waddell, J., H. Steenbock, and E. B. Hart. Wisconsin Agr. Exp. Sta. Bull. 420, p. 72. 1931.
65. Whipple, G. H. and F. S. Robscheit-Robbins. Blood regeneration in severe anemia. VII. Influence of dairy products on hemoglobin production. Amer. Jour. Physiol. **79**: 280-288. 1927.